A study of the delayed type hypersensitivity response to Varicella zoster virus antigen to investigate defects in cutaneous immunity in the elderly

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I, Daisy Sandhu confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

Signed:

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Dedication

This work is dedicated to my parents Rajinder and Sukhwinder Sandhu.

Abstract

Immunity declines with ageing resulting in an increase in incidence and severity of infections such as shingles. We used the delayed type hypersensitivity response as a model for a memory T cell response to investigate differences in the response to VZV antigen administered into the skin between old and young individuals. We found that there is a reduced clinical response, which is associated with a lack of T cell accumulation in the old subjects. The earliest differences between the two groups include reduced activation of dermal endothelium and a reduced infiltration of $CD11c^+$ dendritic cells in the skin of the old individuals after VZV antigen injection. This may reflect a defect in the ability of VZV specific T cells and / or innate immune cells resident in the skin to condition the cutaneous environment adequately to induce an effective adaptive immune response. There is an increased level of Tregs in the normal skin of old individuals and these Tregs may suppress the activation of skin resident cells, thereby reducing early recruitment of memory T cells from the circulation as part of the immune response. Without adequate recruitment and activation of a few VZV specific T cells, we propose there is failure to produce additional mediators needed to amplify the memory response.

Abbreviations

ABC	Avidin and biotinylated horseradish peroxidase macromolecular complex
AEC	3-amino-9-ethyl carbazole
AMP	Adenosine monophosphate
APC	Antigen presenting cell
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin
BDCA	Blood dendritic cell antigen
C. albicans	*Candida albicans*
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CLA	Cutaneous lymphocyte antigen
CMI	Cell mediated immunity
CMV	Cytomegalovirus
cpm	Counts per minute
CTLA	Cytotoxic T-lymphocytes-associated protein
DAMP	Damage associated molecular pattern
DC	Dendritic cell
dDC	Dermal dendritic cell
DC-LAMP	Dendritic cell lysosome-associated membrane protein
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DTH	Delayed type hypersensitivity
EI	Erythema-index
ELISPOT	Enzyme linked immunospot assay
E-selectin	Endothelial cell selectin
GITR	Glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HEV	High endothelial venule

HLA	Human leukocyte antigen
HSV	Herpes simplex virus
HZ	Herpes zoster
ICAM	Intercellular adhesion molecule
IDEC	Inflammatory dendritic epidermal cell
IDO	Indolamin 2,3-dioxygenase
IE	Intermediate early
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iTreg	Induced T regulatory cell
JAM	Junctional adhesion molecule
LC	Langerhans cell
LFA	Leucocyte function-associated antigen
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MT	Mantoux test
M. tuberculosis	*Mycobacterium tuberculosis*
Nrp	Neuropilin
nTreg	Naturally occurring regulatory T cell
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PECAM	Platelet endothelial cell adhesion molecule
PNAd	Peripheral node addressin
PPD	Purified protein derivative
PRR	Pattern recognition receptor
PSGL	P-selectin glycoprotein ligand
P-selectin	Platelet selectin
PV	Perivascular infiltrate
RPMI	Roswell Park Memorial Institute medium

S1P	Sphingosine-1-phosphate
SD	Standard deviation
SEM	Standard error of the mean
T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell
T_{FH}	Follicular helper T cell
T_{RM}	Resident memory T cell
TARC	Thymus and activation regulated chemokine
TCR	T cell receptor
TGF	Transforming growth factor
Tip-DC	TNF and inducible NOS producing dendritic cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TSDR	Treg-specific demethylated region
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
VZV	Varicella zoster virus
WNV	West Nile virus

Publications and Presentations

PUBLICATIONS

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POSTER PRESENTATIONS

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Accumulation of varicella zoster specific CD4+Foxp3+ Tregs after cutaneous antigen challenge in humans.
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Early events in the recall response to varicella zoster virus skin antigen in ageing.
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Early events in cutaneous delayed type hypersensitivity response in ageing.
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Chapter 1. Introduction

1.1 The Skin Immune System

The sixteenth century Italian physician Girolamo Mercuriale, author of what is considered to be the first scientific passage on skin diseases, mentions that there was general consensus among physicians of that time that the only functions of the skin were to provide a protective covering for the body and a receptacle for waste material (Sutton, R. L. 1986). Hippocrates had previously highlighted the skin was needed to bind together individual body parts and Plato had likened it to a fisherman's net (Sutton, R. L. 1986). Today, the skin, a complex organ, is recognised to have multiple functions. Whilst providing a physical and chemical barrier to our external environment it also contains peripheral receptors and nerve endings allowing us to sense our surroundings. Homeostatic functions help regulate body temperature and water loss. The skin is able to utilise sunlight to synthesize Vitamin D3, yet contains melanin pigment to protect against sunlight induced DNA damage. In addition to the physical barrier, the skin houses elements of an elaborate immune system (Bos and Kapsenberg 1986) enabling us to tolerate benign foreign material yet raise an immune response to foreign pathogens. The skin is made up of two main layers, the epidermis and the dermis. These contain cells of the innate and adaptive immune system. These cells may be resident within the skin, recruited from the circulation and some may recirculate through the skin (Table 1.1).

	RESIDENT	RECRUITED	RECIRCULATING
INNATE	Keratinocyte	Monocytes	Natural killer cells
	Endothelial cells Vascular Lymphatic	Granulocytes Basophils Eosinophils Neutrophils	Dendritic cells
	Dendritic cells	Mast cells	
	Mast cells		
	Tissue macrophages		
ADAPTIVE	T lymphocytes	T lymphocytes	T lymphocytes
		B lymphocytes	

Table1.1: Innate and Adaptive Cells of the Skin Immune System (adapted from Bos 2004).

1.2 T cell mediated immune responses

1.2.1 Primary T cell response

A primary T cell response is mediated by naïve T cells that, after leaving the thymus, circulate continuously via blood and lymph, repeatedly entering lymph nodes and other secondary lymphoid tissues (Gowans and Knight 1964; Picker and Butcher 1992). Naïve T cells enter lymph nodes via high endothelial venules (HEVs) that constitutively express homing molecules to recruit lymphocytes. These homing molecules mediate a process of T cell rolling, activation and arrest. Essentially, there is a short-term tethering interaction between L-selectin on the naive T cell with peripheral node addressins (PNAd) on the HEV (Rosen 2004) that brings the CC-chemokine receptor 7 (CCR7) on the T cell into proximity with its ligand CC-chemokine ligand 21 (CCL21) on the HEV. Activation of the integrin lymphocyte function-associated antigen 1 (LFA-1) expressed on the T cell as a result of signalling induced by the binding of CCR7 to CCL21 results in firm engagement

between LFA-1 and intercellular adhesion molecule 1 (ICAM-1) on the HEV causing T cell arrest (Shamri, Grabovsky et al. 2005). These T cells subsequently transmigrate into the T cell zone of the lymph node.

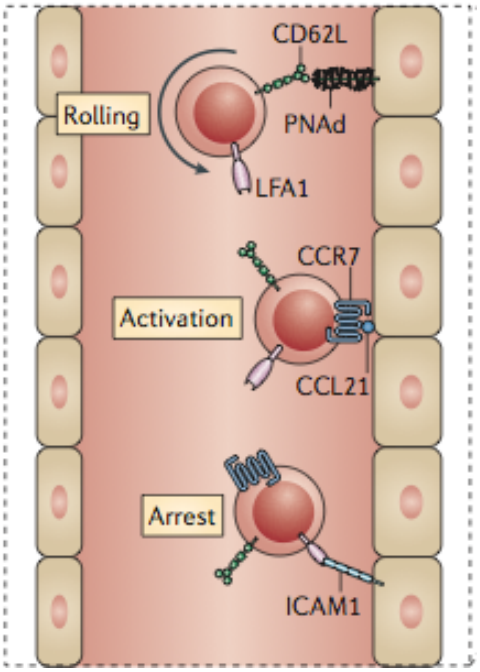


Figure 1.1 T cell migration through high endothelial vessels.
T cells enter lymphoid tissue by crossing the walls of high endothelial vessels (HEVs). There is initial binding between L-selectin (CD62L) on the T cell and peripheral node addressins (PNAd) on the HEV mediating a rolling interaction. Subsequent stimulation of CCR7 by CCL21 bound on the HEV luminal surface activates LFA-1 causing the T cell to bind firmly to ICAM-1 on the endothelial cell resulting in cell arrest. (Adapted from (Masopust and Schenkel 2013).

A primary T cell response is initiated within secondary lymphoid tissue when a naïve $CD4^+$ or $CD8^+$ T cell encounters its specific foreign antigen peptide-major histocompatibility complex (MHC) on an activated antigen presenting cell (APC) (Germain and Stefanova 1999; Itano and Jenkins 2003). Activated APCs that have captured pathogens at peripheral sites migrate to the regional lymph nodes and enter the T cell zone where they present antigen. Activation of a naïve T cell involves the integration of three signals (Kapsenberg 2003).

The first is produced when the peptide-MHC on the APC binds to the T cell receptor (TCR) and co-receptor (either CD4 or CD8). Engagement of co-stimulatory molecules such as CD28 on the T cell with those on the APC provides a second signal, promoting survival and extensive proliferation of the antigen-specific T cell (Blattman, Antia et al. 2002). The third signal provided by cytokines drive differentiation of the cells into one of a variety of effector subsets and directs migration. With the exception of $CD4^+$ follicular helper T cells (T_{FH}) that remain in the secondary lymphoid tissue and help orchestrate humoral responses by interacting with B lymphocytes (King, Tangye et al. 2008), most other T cells leave to locate the site of active infection.

During T cell activation in the secondary lymphoid tissue, T cells are effectively trapped within the T cell zone first to allow antigen sampling and then proliferation of activated cells (Shiow, Rosen et al. 2006). However, as activation wanes, lymphocytes egress in response to a differential in concentration of sphingosine-1-phosphate (S1P), a signalling molecule abundantly expressed in blood and efferent lymph vessels (Cyster and Schwab 2012). Activated T cells exiting the lymphoid tissue are also drawn to areas of inflammation by chemotaxis and may express homing molecules targeting them to non-lymphoid tissues e.g. skin homing T cells may express cutaneous lymphocyte antigen (CLA) which bind E-selectin expressed on activated cutaneous vascular endothelium (Berg, Yoshino et al. 1991); gut homing T lymphocytes express the integrin α4β7 and the receptor CC chemokine receptor 9 (CCR9) that binds to the intestinal mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and the CC chemokine ligand 25 (CCL25) on intestinal epithelial cells respectively (Wurbel, Malissen et al. 2001; Salmi and Jalkanen 2005). There is evidence that suggests expression of tissue homing molecules may reflect the site of T cell priming, for example T cells activated in skin draining lymph nodes display an enhanced capacity to migrate to inflamed skin while those activated in intestine associated lymphoid tissue become CCR9 and α4β7 expressing gut-tropic T cells (Campbell and Butcher 2002; Sigmundsdottir and Butcher 2008; Agace 2010). However, the effect of this imprinting may be transient (Masopust, Choo et al. 2010) may be

influenced by trafficking through other distant lymph nodes (Liu, Fuhlbrigge et al. 2006) and may vary between $CD4^+$ and $CD8^+$ subsets (Gebhardt, Whitney et al. 2011).

At the site of inflammation activated T cells carry out their effector functions. Activated $CD8^+$ T cells all differentiate into cytotoxic T cells capable of killing infected target cells through the secretion of perforins, granzymes, granulysin and cytokines such as interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α). In contrast $CD4^+$ T cells differentiate into one of several effector subsets e.g. T_H1, T_H2, T_H17 T_{FH}, induced T regulatory cells (iTreg) (Mosmann, Cherwinski et al. 1986; Yamane and Paul 2012). This is not a complete list of effector subsets and the number of subsets will presumably increase as effector T cells become better characterised allowing us to more accurately distinguish between them. Although commitment to one of these lineages may begin during T cell activation in the secondary lymphoid tissue, it is recurrent exposure to antigen and polarising cytokines that determines further differentiation (Bajenoff, Wurtz et al. 2002). IL-12 promotes the development of T_H1 polarised cells that secrete IFN-γ while IL- 4 promotes the development of T_H2 cells that typically secrete IL-4, IL-5 and IL13. IL-6 and transforming growth factor-beta (TGF-β) promote development of T_H17 cells that produce IL-17A, IL-17F, TNF-α, IL-21 and IL22 and depend upon IL-23 for their development, survival and proliferation (Harrington, Hatton et al. 2005; Park, Li et al. 2005). T_H17 are highly pro-inflammatory and help protect against extracellular bacteria and fungi. T_H1 cells generally help clear intracellular pathogens while T_H2 cells respond to parasitic pathogens. iTregs can be generated in the presence of TGF-β and are discussed in more detail below.

After an adequate primary immune response to an infection has been mounted such that the effector T cells have removed the stimulus that originally recruited them, the majority of T cells undergo apoptosis. However a small proportion of antigen experienced cells persist and provide a memory population for future encounters.

1.2.2 Memory T cell response

Memory or recall responses occur more rapidly and are more pronounced than primary responses (Ahmed and Gray 1996). This may in part due to the increased frequency of antigen specific cells, but it is also thought that memory T cells have reduced activation requirements, are able to respond to lower concentrations of antigen and require a shorter duration of antigenic stimulation (Berard and Tough 2002). Memory cells may also proliferate more rapidly following TCR stimulation and display faster kinetics of cytokine secretion (Rogers, Dubey et al. 2000; Veiga-Fernandes, Walter et al. 2000; Berard and Tough 2002). Memory cells also display altered patterns of adhesion molecule expression and migration (Masopust, Vezys et al. 2001).

Circulating memory T cells have largely been divided into two functionally distinct subsets (Sallusto, Lenig et al. 1999; Sallusto, Geginat et al. 2004). Central memory T (T_{CM}) cells are $CD62L^{+}CCR7^{+}$, typically migrate between blood and secondary lymphoid tissues akin to naïve T cells, are able to produce ample IL-2 and proliferate extensively in response to antigen stimulation and will eventually demonstrate effector functions. In contrast effector memory T (T_{EM}) cells are typically $CD62L^{-}CCR7^{-}$, circulate between blood and non-lymphoid tissues, have less proliferative capacity but rapidly secrete effector cytokines in response to antigen stimulation. However, although CCR7 expression is a cardinal feature of T_{CM} cells, CCR7 expression has also been found on memory cells within non-lymphoid tissues and appears to be required for emigration from these peripheral tissues (Campbell, Murphy et al. 2001; Bromley, Thomas et al. 2005; Debes, Arnold et al. 2005). Several models have been proposed to reflect the relationship between these two memory subsets. While some groups suggest that the two subsets are interdependent and that T_{CM} cells may differentiate into T_{EM} cells (Lanzavecchia and Sallusto 2000), or that T_{EM} cells convert to T_{CM} cells over time (Wherry, Teichgraber et al. 2003; Marzo, Klonowski et al. 2005), others suggest that they may represent two separate lineages (Baron, Bouneaud et al. 2003).

Recently, a tissue resident subset of T_{EM} (T_{RM}) cells that does not recirculate has been identified in various organs in animal studies (see Table 1.2) (Bevan 2011). Although most studies have shown these to be $CD8^+$ T cells populations (Gebhardt, Wakim et al. 2009; Masopust, Choo et al. 2010; Wakim, Woodward-Davis et al. 2010; Gebhardt, Whitney et al. 2011; Jiang, Clark et al. 2012), a long lived $CD4^+$ T cell population has been detected in mouse lung (Teijaro, Turner et al. 2011). These T_{RM} cells have, in some cases shown to provide superior protection compared to the circulating memory pool and as such may provide an important role in first line defence (Gebhardt, Wakim et al. 2009; Teijaro, Turner et al. 2011; Jiang, Clark et al. 2012). Heterogeneity within the T_{RM} populations in different organs may reflect tissue specific signals, although several studies do highlight enhanced expression of CD69 and CD103 (Gebhardt, Wakim et al. 2009; Wakim, Woodward-Davis et al. 2010; Gebhardt, Whitney et al. 2011; Jiang, Clark et al. 2012). CD103 is an integrin expressed at intermediate levels on naïve T cells, with expression down regulated upon activation. Memory T cells maintain a low level of expression. CD103 binds E-cadherin, an adhesion molecule found on epithelial cells (Cepek, Shaw et al. 1994). It remains to be determined whether CD69 acts to prevent tissue egress as is seen with naïve T cells during activation in secondary lymphoid tissue (Shiow, Rosen et al. 2006).

There is evidence supporting the existence of such resident, non-recirculating memory T cells in human skin. After treatment with low-dose alemtuzumab (anti-CD52) to treat T cell malignancies, some T cells persisted in the skin despite depletion of T cells from the blood (Clark, Watanabe et al. 2012). Xenografting of non-lesional human skin from a patient with psoriasis to severely immunocompromised mice results in active disease phenotype in the graft (Boyman, Hefti et al. 2004). Furthermore intra-epidermal $CD8^+$ T cells have shown to persist for years in fixed drug eruption lesions in the absence of repeat antigen challenge (Mizukawa, Yamazaki et al. 2008).

SUBSET	PHENOTYPE	LOCATION
T_{CM}	CD62L+, CCR7+, CD69-, CD103-	Lymph nodes, spleen (white pulp > red pulp), blood and bone marrow
T_{EM}	CD62L-, CCR7-, CD69-, CD103-	Spleen (red pulp > white pulp); fewer in lymph nodes, blood, lung, liver, intestinal tract, reproductive tract, kidney, adipose tissue and heart
T_{RM}	CD62L-, CCR7-, CD69+, CD103+	CD8: epithelium of the skin, gut, and vagina; salivary glands and lung airways; brain and ganglia CD4: (?) lung parenchyma

Table 1.2 Memory T cell subsets (Adapted from (Mueller, Gebhardt et al. 2013)

Naïve, primed and memory T cell subsets can be distinguished by expression of CD45, the leukocyte common antigen (Trowbridge and Thomas 1994) that constitutes approximately 10% of the lymphocyte cell surface (Thomas 1998). CD45 is a tyrosine phosphatase that regulates signalling through antigen receptors and exists in multiple isoforms. Naïve T cells express CD45RA, the highest molecular weight isoform, but this is rapidly lost during activation and the low molecular weight CD45RO isoform is up-regulated (Akbar, Terry et al. 1988). CD45RO can be divided into CD45RB bright or dull cells and progressive differentiation of CD45RO cells is accompanied by a decline in CD45RB expression (Salmon, Pilling et al. 1994). Within the CD45RA and CD45RO populations, expression of the co-stimulatory molecules CD27 and CD28 can be used to identify more or less differentiated cells (Romero, Zippelius et al. 2007).

However, studies have shown that some subsets of effector T cells can re-express CD45RA and that these cells may be close to end-stage differentiation (Hamann, Kostense et al. 1999; Koch, Larbi et al. 2008). Age and the presence of CMV infection have shown to contribute to the accumulation of these CD45RA re-expressing cells (Koch, Larbi et al. 2008;

Chidrawar, Khan et al. 2009; Libri, Azevedo et al. 2011). Re-expression of CD45RA on memory cells may be driven by homeostatic cytokines in the absence of antigen (Geginat, Lanzavecchia et al. 2003; Dunne, Belaramani et al. 2005; Griffiths, Riddell et al. 2013). The purpose of these CD45RA expressing effector T cells remains uncertain but it has been proposed that they represent a pool of short-lived potent effector cells (Henson, Riddell et al. 2012).

1.2.3 Delayed Type Hypersensitivity Response in the Skin

A delayed type hypersensitivity (DTH) response is an in vivo manifestation of cell-mediated immunity (CMI) (Turk 1980; Landsteiner and Chase 1942). It is a useful experimental system to study memory responses in the skin mediated by antigen-specific T cells in an immune individual. Delivery of the antigen may be epicutaneous or via intradermal inoculation.

The DTH skin test can be used clinically to determine infection with or prior exposure to a pathogen. The Mantoux test (MT) is a classical DTH response whereby purified protein derivative (PPD) from *Mycobacterium tuberculosis* (*M. tuberculosis*) is injected intradermally. A positive response in an immunocompetent individual indicates immunity acquired through natural infection or from the Bacille Calmette-Guerin (BCG) vaccine (Ahmed and Blose 1983). DTH responses to common pathogens such as *Candida albicans* (*C. albicans*) can be used to give a general measure of cell-mediated immunity in potentially immunocompromised patients (Ahmed and Blose 1983). DTH responses to the varicella zoster virus (VZV) antigen skin test used in this study have been used previously to identify non-immune, susceptible young children during an outbreak of varicella in an institution (Kamiya, Ihara et al. 1977; Baba, Yabuuchi et al. 1978) and to assess the level of cell mediated immunity in immune adults to determine their risk of VZV reactivation and shingles (Okuno, Takao et al. 2013).

Clinically, positive skin DTH reactions give rise to an area of erythematous induration at the site of antigen challenge, typically on the forearm in humans and the ear or footpad in mice (Turk 1980). Although some changes in the skin may be clinically evident just a few hours after antigen challenge, the clinical response typically peaks at 48 -72 hours in humans and at 24 hours in mice (Ahmed and Blose 1983)(Turk 1980). After the peak, the clinical response resolves over the following 10 -14 days (Turk 1980).

Histologically, the cutaneous DTH response to tuberculin PPD has been well characterised and is bi-phasic with an early non-specific response followed by a second specific response only seen in sensitized individuals (Boughton and Spector 1963; Platt, Grant et al. 1983). The first phase consists of 'danger signals' thought to be provided by cells of the innate immune system in response to the antigen and mechanical trauma associated with its delivery (Matzinger 2002). These signals are thought to facilitate the recruitment of leucocytes to mediate the specific adaptive response (Kupper and Fuhlbrigge 2004). The early infiltrate, within 6 hours after antigen injection, contains numerous neutrophils that decline in number thereafter, with a similar response seen in control subjects (Platt, Grant et al. 1983). Perivascular mononuclear cells are evident 12 hours after antigen injection and both T cell and macrophage numbers increase rapidly peaking at 24-48 hours, with a predominance of T cells (Poulter, Seymour et al. 1982; Platt, Grant et al. 1983; Chu, Field et al. 1992). Only very few B lymphocytes are seen in the response (Poulter, Seymour et al. 1982; Platt, Grant et al. 1983; Gibbs, Ferguson et al. 1984). $CD4^+$ T cells outnumber $CD8^+$ T cells at a ratio of 2:1 throughout the response and although initially perivascular in distribution, after 24 hours T cells can be seen more diffusely dispersed in the dermis, including around adnexal structures such as sweat glands, and in the epidermis (Poulter, Seymour et al. 1982; Platt, Grant et al. 1983; Gibbs, Ferguson et al. 1984; Chu, Field et al. 1992). The mechanism of this cellular infiltration remains poorly defined however innate responses are able to activate signalling pathways that regulate the expression of endothelial adhesion molecules, chemokines and cytokines. These in turn are able to recruit both

non-specific leukocytes such as neutrophils and antigen-specific memory T cells (Kupper and Fuhlbrigge 2004). However, since non-immune individuals do not raise a DTH response despite equal exposure to antigen and trauma to the skin, particular events must be induced to engage antigen-specific memory T cells in immune individuals.

1.2.4 Initiation of a DTH response in skin

A memory response, like a primary T cell response requires antigen to be presented to the memory T cell. Dendritic cells (DCs), are professional APCs and have classically been defined by their ability to capture and process antigen in peripheral tissues where they reside and undergo maturation before migrating to secondary lymphoid organs where they activate naïve T cells (Banchereau and Steinman 1998). The major resident DCs in human skin are Langerhans cells (LCs) that reside in the epidermis and dermal dendritic cells (dDCs) that reside in the dermis. However, the relative contribution of these subsets in antigen presentation of intradermally injected antigen as part of a recall response in humans is not well defined. It has been shown in mice models that both LCs and dDCs are able to take up ovalbumin protein delivered to the intradermal compartment and transport the antigen to the regional lymph nodes (Flacher, Tripp et al. 2010; Sparber, Tripp et al. 2010). It is proposed that the antigen enters the epidermal compartment by diffusion. However ablation of LC's does not diminish the subsequent cytotoxic response suggesting that the dDCs play a more crucial role (Flacher, Tripp et al. 2012). Although most dDCs in normal skin are relatively immature, there is a small subgroup (~5%) expressing mature DC markers and this population may mediate rapid antigen presentation to local T cells (Zaba, Krueger et al. 2009). In addition memory T cells are less stringent in their antigen-presenting requirements compared to naïve T cells (Croft, Bradley et al. 1994) such that skin resident cells e.g., macrophages (Geppert and Lipsky 1989) and keratinocytes (Black, Ardern-Jones et al. 2007) that are capable of presenting antigen may also play a prominent role in initiating a local recall response.

Furthermore, there is mounting evidence that memory immune responses can be initiated in the skin without the need for initial migration of DCs to the lymph nodes. It has been demonstrated that normal human skin contains approximately $1x10^6$ T cells per cm^2 i.e. the skin surface of a normal adult would contain approximately 20 billion T cells, nearly twice the number seen in the entire blood volume (Clark, Chong et al. 2006). More than 90% of CLA^+ T cells were present in the skin. These skin populating T cells were predominantly of a T_H1 effector phenotype and demonstrated a diverse TCR repertoire (Clark, Chong et al. 2006). In support of this diverse TCR repertoire, studies have shown that primed T cells with skin homing molecules are distributed to all parts of the skin although the highest numbers are present at the site of initial pathogen exposure (Gebhardt, Wakim et al. 2009; Jiang, Clark et al. 2012). Indeed studies in mice have confirmed that T cells can become activated, proliferate and carry out effector functions locally in non-lymphoid organs (Wakim, Waithman et al. 2008; Jiang, Clark et al. 2012).

A locally initiated memory response in humans would be in keeping with the finding that changes are seen within a few hours in the skin after secondary antigen challenge yet it is estimated it would take 12 hours for antigen in the toe to travel to the inguinal lymph nodes (Mestas and Hughes 2004). In addition, recall responses can occur in spite of compromised afferent lymphatics in patients with lymphoedema (Mallon, Powell et al. 1997).

Therefore, there are three ways by which antigen-specific T cells appear in the skin. Firstly, antigen-specific T_{RM} and those T_{EM} cells already present in the skin could become activated by antigen presented by one of several potential APCs. Secondly, activation of dermal endothelium could recruit both antigen specific and non-specific circulating T_{EM} cells into the skin. Thirdly antigen laden dendritic cells could migrate to regional lymph nodes and present antigen to T_{CM} cells, resulting in their proliferation and differentiation into antigen specific skin homing effector cells able to reinforce the early DTH response. This concept is illustrated in Figure 1.2.

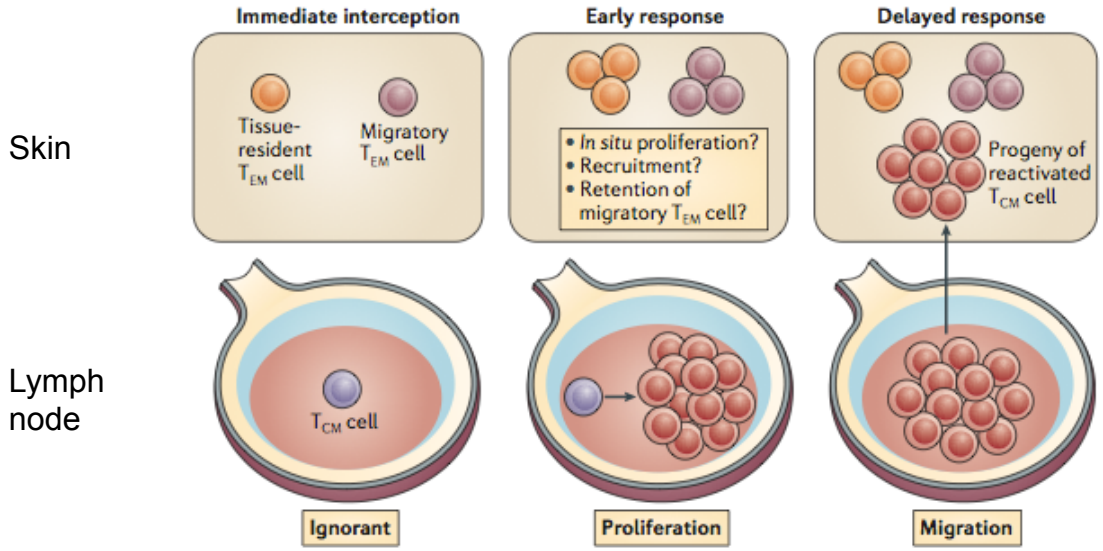


Figure 1.2 Dynamics of the memory T-cell recall response in peripheral tissue.

Recall responses may incorporate 3 tiers of responding cells. The first, T_{RM} or T_{EM} cells within the skin, may proliferate and exert effector functions after local antigen presentation. Inflammation, leading to endothelial activation would allow recruitment of both specific and non-specific T_{EM} cells that would display rapid effector functions upon activation. Finally antigen-laden APCs migrating to regional lymph nodes would present antigen to T_{CM} cells with subsequent proliferation and differentiation into skin homing effector cells. (Figure adapted from (Masopust and Schenkel 2013)).

1.2.5 T cell migration to the skin

In order to recruit circulating T cells into the skin during a secondary memory immune response, both T cells and the activated endothelium of dermal post-capillary venules must express corresponding adhesion molecules to allow T cells to adhere firmly to the endothelium before transmigrating into the skin (von Andrian and Mackay 2000). The important contribution made by recruitment of circulating T cells has been shown in mouse studies where blocking chemokine or integrin mediated T cell recruitment results in a

diminished DTH response (Reiss, Proudfoot et al. 2001; Grabbe, Varga et al. 2002).

T cell migration consists of three stages, selectin-mediated rolling, chemokine-triggered activation and integrin-dependent arrest. Transmigration into the tissue usually occurs in a paracellular manner but can be by the transcellular route mediated by CD31 (platelet endothelial cell adhesion molecule, PECAM-1), CD99 and junctional adhesion molecule (JAM) (Ley, Laudanna et al. 2007). The adhesion molecules that mediate the process of migration are shown in table 1.3.

	Lymphocyte adhesion molecule	**Endothelial adhesion molecule**
Rolling	CLA PSGL-1	E-selectin P-selectin
Arrest	LFA-1 VLA-4	ICAM-1 VCAM-1
Transmigration	CD31 (PECAM-1) Junctional adhesion molecule; LFA-1, VLA-4	CD31 (PECAM-1) Junctional adhesion molecule

Table 1.3 Adhesion molecules mediating T cell migration into inflamed skin

1.2.5.1 Leucocyte rolling

Leucocyte rolling is mediated by a weak, impermanent interaction between selectin molecules and their ligands. Endothelial cells constitutively synthesise and store P-selectin, releasing it within minutes to the cell surface upon activation. However, it is the expression of E-selectin in response to pro-inflammatory cytokines such as TNF-α, interleukin 1 (IL-1) (Bevilacqua 1993) and IFN-Y (Lee, Chung et al. 1995) that is considered to be important in

recruiting memory cells into the skin during inflammation. Memory T cells express P-selectin glycoprotein ligand 1 (PSGL-1) to allow entry into to peripheral sites of inflammation. Skin homing memory T cells also express CLA, a modified PSGL-1 molecule that facilitates binding to E-selectin (Berg, Yoshino et al. 1991; Fuhlbrigge, Kieffer et al. 1997). The rolling motion that results from these interactions is slower than that of free-flowing cells and allows sampling of the endothelial surface for activating molecules such as chemokines.

1.2.5.2 Leucocyte activation and arrest

To stop rolling, T cells must engage additional receptors of the integrin family. Memory T cells express the integrins LFA-1 and very late antigen-4 (VLA-1), which bind ICAM-1 and VCAM-4 (vascular cell adhesion molecule-4) expressed on activated endothelium respectively (von Andrian and Mackay 2000). However, to mediate cell arrest, these integrins need to receive activating signals. These intracellular activating signals occur in response to the T cell binding chemokines expressed on the endothelial surface. Activated integrins undergo a conformational change that results in a higher binding affinity with their ligands decreasing the rate of ligand dissociation (Ley, Laudanna et al. 2007). Chemokines are secreted polypeptides that may be synthesized and/or presented by activated endothelial cells on their luminal surface or in the extracellular matrix to trigger intravascular cell arrest or direct the migration of T cells (Campbell, Hedrick et al. 1998). Several chemokine receptors e.g. CCR4, CCR10, CCR6 and CCR8 may play a role in recruiting T cells into the skin though CCR4 appears to play a central role (Campbell, Haraldsen et al. 1999; Fitzhugh, Naik et al. 2000; Reiss, Proudfoot et al. 2001; Campbell, O'Connell et al. 2007; Islam, Chang et al. 2011). CLA^+ T cells typically co-express CCR4 in the skin and to a lesser degree in the circulation (Clark, Chong et al. 2006). The CCR4 ligand, thymus and activation regulated chemokine (TARC) is constitutively expressed at low levels in the skin and expression can be up-regulated in cutaneous inflammation (Saeki and Tamaki 2006).

1.2.6 T cell proliferation during a cutaneous DTH response

During a DTH response in the skin there is an initial accumulation of T cells followed by a decline in T cell numbers (Poulter, Seymour et al. 1982). This accumulation may in part result from proliferation of antigen-specific T cells in the skin, which are either resident or have been recruited from the circulation. It has been shown in mice during a memory $CD8^+$ T cell response to HSV, that tissue-resident antigen-specific T cells can undergo stimulation and proliferation in response to local infection in the dorsal root ganglia (Wakim, Waithman et al. 2008). The same group has also shown that during the memory response, both antigen specific and non-specific $CD8^+$ T cells are recruited to the dorsal root ganglia and that there is subsequent local expansion of the antigen-specific T cells through proliferation rather than simply preferential accumulation and retention of circulating antigen-specific T cells (Wakim, Gebhardt et al. 2008). Published work from our group also suggests that there is *in situ* proliferation of antigen-specific T cells during the Mantoux response in humans although we were unable to distinguish if this was proliferation of resident or recruited T_{EM} cells (Vukmanovic-Stejic, Agius et al. 2008).

1.2.7 Regulatory T cells and their role in a cutaneous DTH response

Regulatory T cells (Tregs) are a subset of T cells that exert a suppressive or regulatory role, important in immunological self-tolerance and resolution of immune responses to foreign antigens (Sakaguchi 2004). Tregs were identified within a subset of $CD25^+$ T cells that make up 5-10% of peripheral $CD4^+$ T cells and less than 1% of peripheral $CD8^+$ T cells in naive mice and humans (Sakaguchi, Sakaguchi et al. 1995; Sakaguchi 2004). The importance of Tregs was demonstrated in experiments where depletion of this $CD25^+$ subset from mice was sufficient to activate self-reactive T cells and elicit autoimmune disease (Sakaguchi, Sakaguchi et al. 1995). Foxp3 is a transcription factor expressed by Tregs and is required for Treg development (Ohkura, Kitagawa et al. 2013). Mutations of human *Foxp3* result in impaired

Treg function and immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) that manifests with severe autoimmune diseases, inflammatory bowel disease and allergy (Bennett, Christie et al. 2001; Bacchetta, Passerini et al. 2006). Mice with mutations in the *Foxp3* gene develop fatal lymphoproliferative disorder due to a deficiency of Tregs (Brunkow, Jeffery et al. 2001; Fontenot, Gavin et al. 2003). Foxp3 had been proposed as a Treg lineage specific factor (Fontenot, Rasmussen et al. 2005) but it was later shown that activated conventional $CD4^+$ T cells may temporarily up-regulate expression of Foxp3 without acquisition of Treg function (Gavin, Torgerson et al. 2006; Allan, Crome et al. 2007; Wang, Ioan-Facsinay et al. 2007).

Naturally occurring Tregs (nTregs) are produced by the thymus (Itoh, Takahashi et al. 1999). Naïve nTregs are $CD45RA^+$ (Valmori, Merlo et al. 2005) and are thought to circulate in a manner akin to conventional naïve T cells, being activated in regional lymph nodes (Sakaguchi, Yamaguchi et al. 2008). Like conventional T cells, activated nTregs give rise to a $CD45RO^+$ memory nTreg population (Miyara, Yoshioka et al. 2009; Booth, McQuaid et al. 2010). nTregs generated in the thymus are widely thought to be reactive for self-antigens, having escaped deletion and instead acquired suppressive function (Jordan, Boesteanu et al. 2001; Hsieh, Lee et al. 2012). However, Treg populations can also be generated in the periphery from conventional $CD4^+$ T cells during both inflammatory and non-inflammatory conditions and these are referred to as adaptive or induced Tregs (iTregs) (Bilate and Lafaille 2012). In keeping with this it has been shown that Tregs maintain a highly diverse TCR repertoire that include most of the TCRs dominantly expressed by naïve T cells, and that the frequency of TCRs with high affinity for self- versus non-self antigens is similar within the $CD4^+Foxp3^+$ Treg and $CD4^+Foxp3^-$ naïve T cell populations (Pacholczyk, Kern et al. 2007).

Tregs can be identified by their expression of Foxp3 although as mentioned above Foxp3 is also transiently expressed on activated T cells. However, a Treg-specific demethylated region (TSDR) at the *foxp3* locus in nTregs,

relates to stability of Foxp3 expression (Floess, Freyer et al. 2007) and in combination with Foxp3 identifies cells committed to Treg cell lineage (Ohkura, Hamaguchi et al. 2012). However, the intracellular location of these markers limits their use to isolate Tregs for further analysis.

Surface markers used to identify Tregs include: high levels of CD25, cytotoxic T-lymphocytes-associated protein 4 (CTLA-4), an inhibitory receptor which competes with CD28 for B7 ligands expressed on activated DCs, and glucocorticoid-induced TNFR-related protein (GITR), a member of the TNF receptor superfamily. Both CTLA-4 and GITR are constitutively expressed on nTregs whereas expression of these receptors is only up-regulated on conventional T cells after activation. CD127, the alpha chain of the IL-7 receptor, is often used in conjunction with CD25 to distinguish between $CD25^+$ activated conventional T cells and Tregs since expression of CD127 is high on activated conventional T cells and low on Tregs (Liu, Putnam et al. 2006; Seddiki, Santner-Nanan et al. 2006). CD39, an ectonucleotidase found on nTregs responsible for cleaving adenosine triphosphate (ATP) to adenosine monophosphate (AMP) (Borsellino, Kleinewietfeld et al. 2007; Deaglio, Dwyer et al. 2007) has also been used in combination with $CD25^+$ to more accurately isolate Treg populations (Mandapathil, Lang et al. 2009; Schuler, Harasymczuk et al. 2011) although CD39 is also expressed on multiple other activated immune cells. Expression of Helios, an Ikaros transcription family member was thought to be limited to nTregs (Thornton, Korty et al. 2010), however others report that Helios may be expressed on *in vitro* generated iTregs (Verhagen and Wraith 2010) as well as activated $CD4^+$ T cells (Akimova, Beier et al. 2011). Similarly neuropilin 1 (Nrp 1), a receptor for members of the vascular endothelial growth factor (Soker, Takashima et al. 1998) and semaphorin family (Kolodkin, Levengood et al. 1997) has been used to distinguish Tregs (high expression) from activated T cells (low expression) (Bruder, Probst-Kepper et al. 2004) and had been proposed as a marker to help distinguish nTregs from *in vivo* generated iTregs (Yadav, Louvet et al. 2012). However, while iTregs generated in tolerogenic environments are largely Nrp-1^{lo}, Nrp-1 is expressed on iTregs generated in

inflammatory conditions (Weiss, Bilate et al. 2012). The lack of a specific marker to distinguish between Tregs and activated T cells means a combination of markers is often used in functional studies and differences in the markers used may give rise to some discrepancies in data generated by different groups. The lack of a marker to differentiate between nTregs and iTregs means it is not currently possible to determine if Tregs are definitively of nTreg or iTreg origin *in vivo* in humans.

Although Tregs are activated in an antigen specific manner, they are able to carry out their effector functions in an antigen non-specific manner (Takahashi, Kuniyasu et al. 1998). Tregs can suppress various stages of immune responses by targeting effector T cells as well as APCs (Vignali, Collison et al. 2008) using both contact dependent and contact independent mechanisms, although the exact mechanisms of suppression remain unclear. Contact dependent mechanisms include physical interference preventing antigen-specific naïve T cells from accessing APCs as a result of preferential binding of Tregs to APCs compared to naïve T cells (Onishi, Fehervari et al. 2008; Yokosuka, Kobayashi et al. 2010); use of the cytotoxic molecules granzyme and perforin to induce T cell or DC apoptosis (Cao, Cai et al. 2007; Boissonnas, Scholer-Dahirel et al. 2010); upregulation of the tryptophan catabolising enzyme indolamin 2,3-dioxygenase (IDO) on DCs which has immunoregulatory effects and can hinder the activation of T cells (Fallarino, Grohmann et al. 2003); expression of CD39, a nucleoside triphosphate diphosphohyrodolase-1 and CD73, an ecto-5'-nucleotidase (Kobie, Shah et al. 2006; Deaglio, Dwyer et al. 2007) which help generate adenosine, a potent inhibitor of T cell responses (Huang, Apasov et al. 1997). Contact independent mechanisms include IL-2 sequestration from the environment ahead of conventional antigen-specific T cells (Thornton and Shevach 1998; O'Gorman, Dooms et al. 2009; Busse, de la Rosa et al. 2010) and release of immune suppressive cytokines such as TGF-β and IL-10 (Fahlen, Read et al. 2005; Kearley, Barker et al. 2005; Strauss, Bergmann et al. 2007; Rubtsov, Rasmussen et al. 2008). The mechanisms used are likely to vary according

to whether Tregs are acting to provide immune tolerance or immune regulation (Yamaguchi, Wing et al. 2011).

Tregs make up approximately 5-15% of the T cell population resident in normal skin (Clark and Kupper 2007; Agius, Lacy et al. 2009) and the vast majority of these Tregs express $CD45RO^+$ corresponding to a memory Treg population (Booth, McQuaid et al. 2010). This significant presence of memory Tregs in the skin is not surprising given that the skin is the interface with the external environment and must mediate both tolerance to harmless and self antigens in the absence of inflammation and mount immune responses to invading pathogens. Circulating Tregs express high levels of skin homing molecules, with uniform expression of CCR4, 80% expressing CLA and 73% expressing CCR6 (Hirahara, Liu et al. 2006). This expression of skin homing molecules on circulating Tregs is more pronounced than on circulating $CD4^+$ non-Treg T cells. The importance of Tregs being able to access the skin even in the absence of inflammation is highlighted in a study where loss of fucosyltransferase VII enzyme which leads to generation of part of the CLA molecule, dramatically reduced Treg accumulation in the skin, and resulted in onset of severe cutaneous inflammation (Dudda, Perdue et al. 2008). In the absence of inflammation, it has been shown that LCs induce the activation and proliferation of skin resident Tregs and this is thought to play a role in the maintenance of tolerance in normal skin (Seneschal, Clark et al. 2012). During an *in vivo* recall response to PPD in human skin our group has shown that Tregs proliferate *in situ* and accumulate with kinetics very similar to those of conventional memory $CD4^+$ T cells (Vukmanovic-Stejic, Agius et al. 2008). This fine balance between theses two populations and their lifelong maintenance is presumably crucial in regulating the intensity and resolution of memory responses (Akbar, Vukmanovic-Stejic et al. 2007).

1.3 Cellular innate immune system in the skin

Cells of the innate immune system also reside in or are recruited into the skin and compose the early innate immune response. These include

monocytes/macropahges, polymorphonuclear leukocytes, dendritic cells and NK cells (Goodarzi, Trowbridge et al. 2007). These cells have pattern recognition receptors (PRRs) that recognise pathogen-associated molecular patterns (PAMPS), molecules typical of a microbes and damage associated molecular patterns (DAMPs), typically host-derived intracellular molecules (Matzinger 2002; Medzhitov and Janeway 2002; Kono and Rock 2008). PRRs mediate directed phagocytosis, activate inflammatory signalling pathways, induce cell death and activate complement or coagulation cascades (Janeway and Medzhitov 2002; Clark and Kupper 2005). Toll-like receptors (TLR) were the first PRRs to be identified and have been well characterised in humans. TLRs are expressed on both innate immune cells and on other cells such epithelial cells, endothelial cells and fibroblasts (Clark and Kupper 2005). Where the innate immune response cannot contain an infection, it instructs and directs an adaptive immune response.

1.3.1 Macrophages

Macrophages, derived from circulating monocytes, are a tissue resident population (van Furth and Cohn 1968). They use PRRs to recognise and subsequently phagocytose and eliminate pathogens (Aderem and Underhill 1999). In response to pathogen binding macrophages release pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, TNF-α and the chemokine CXCL8 to induce a state of inflammation (Janeway 2012). Such inflammation activates local endothelium to express adhesion molecules that promote the recruitment of other effector leucocytes. Activated macrophages are also able to process and present antigen via MHC class II molecules to primed effector $CD4^+$ T cells (Underhill, Bassetti et al. 1999). Macrophage activation may be influenced by the environmental cytokine milieu during an immune response. IFN-γ primes macrophages to undergo classical activation and develop a harsh pro-inflammatory response while IL-4 and IL-13 promote alternative activation of macrophages (Martinez 2011). Alternatively activated macrophages are associated with the synthesis of anti-inflammatory and tissue repair factors (Van Dyken and Locksley 2013).

1.3.2 Dendritic Cells

1.3.2.1 Langerhans Cells (LCs)

Langerhans cells reside in the suprabasal layers of epidermis. They are thought to be continuously replaced from a resident precursor pool throughout life under steady-state conditions but during inflammation when LCs migrate out of the skin in large numbers, LCs are most likely repopulated by blood borne mononcytes (Merad, Manz et al. 2002; Ginhoux, Tacke et al. 2006). Classically, LCs were thought to perform an immunostimulatory role by capturing and processing cutaneous antigens, migrating to draining lymph nodes and presenting antigen to activate T cells (Banchereau and Steinman 1998). However, recent observations suggest that while they generate T cell effector responses during skin infection, in the steady state LCs mediate tolerance presumably to commensal flora by inducing proliferation of skin resident Tregs (Seneschal, Clark et al. 2012).

1.3.2.2 Dermal dendritic cells (dDC)

These are several DC subsets resident in the dermis of human skin but there is no single or specific marker for them and they are currently best identified by the integrin CD11c (Zaba, Fuentes-Duculan et al. 2007). The major DC population in the dermis of the skin is blood dendritic cell antigen 1 positive (BDCA-1$^+$) (Zaba, Krueger et al. 2009) also known as CD1c. Members of the CD1 family are able to present lipid antigens such as those from *M. tuberculosis* (Cohen, Garg et al. 2009). In the steady state these BDCA-1$^+$ DCs are relatively immature with moderate T-cell stimulatory ability but this is greatly enhanced with DC maturing stimuli (Zaba, Fuentes-Duculan et al. 2007). A proportion of these BDCA-1$^+$ DCs also express CD1a (Zaba, Krueger et al. 2009) and CCR7 and exhibit a chemotactic response to the lymph node chemokine CCL19, compatible with a migratory role to draining lymph nodes for antigen presentation (Angel, George et al. 2006). Other

minor resident dermal dendritic cell populations have also been identified. BDCA-3$^+$ DCs constitute approximately 10% of all CD11c$^+$ DCs (Zaba, Krueger et al. 2009) and are able to cross present antigen to CD8$^+$ T cells *in vitro* (Haniffa, Shin et al. 2012). BDCA-1$^+$ and BDCA-3$^+$ dDCs may be derived from their circulating counterparts (Autissier, Soulas et al. 2010). A less-well defined monocyte or macrophage-like CD14$^+$ population which may arise from monocytes is also present in the dermis of normal skin (Angel, Lala et al. 2007; Collin, McGovern et al. 2013).

During cutaneous inflammation, additional 'inflammatory' dermal dendritic cells appear. These include TNF and inducible NOS producing DCs (Tip-DCs) found in psoriasis (Lowes, Chamian et al. 2005) and inflammatory dendritic epidermal cells (IDECs) found in atopic dermatitis (Guttman-Yassky, Lowes et al. 2007). It is unclear whether these are derived from circulating DC precursors, monocytes or resident DCs (Zaba, Krueger et al. 2009) and whether they have the ability to migrate to lymph nodes (Collin, McGovern et al. 2013).

1.3.2.3 Plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDCs) are characterised by their ability to rapidly make vast quantities of type 1 interferon during viral infections. They make 200-1000 times more type I interferon than any other blood cell type in response to viral PAMPs binding to their PRRs (TLR7 and TLR9) (Siegal, Kadowaki et al. 1999; Gilliet, Cao et al. 2008). Although pDCs are able to present antigen to T cells, their primary role is thought to be in antiviral defence. pDCs circulate in the blood, representing 0.5% of peripheral blood mononuclear cells in humans (Auffray, Sieweke et al. 2009). They are rarely present in normal skin of healthy adults (Zaba, Krueger et al. 2009) (Ebner, Ehammer et al. 2004; Nestle, Conrad et al. 2005) but are thought to play a role in the development of skin lesions in patients with psoriasis (Nestle, Conrad et al. 2005) . They lack the surface marker CD11c but exclusively express BDCA-2 (Dzionek, Fuchs et al. 2000).

1.3.3 Neutrophils

Unlike macrophages and dendritic cells, neutrophils are not present in healthy tissue but are typically the first leukocyte population to be recruited during acute inflammation accumulating in large numbers (Peters, Egen et al. 2008; Basran, Jabeen et al. 2013). Neutrophils make up 50-70% of circulating leukocytes (Mestas and Hughes 2004). They are very short lived with a half-life of 8-12 hours in the circulation (Dancey, Deubelbeiss et al. 1976) although this is increased several fold during inflammation in response to cytokines, growth factors and bacterial products (Colotta, Re et al. 1992). During maturation, granules form inside the neutrophil and contain pro-inflammatory proteins. Neutrophils are able to mediate both intracellular and extracellular elimination of pathogens e.g., they can release the contents of their granules either into phagosomes or into the extracellular milieu (Kolaczkowska and Kubes 2013).

Murine studies have identified distinct neutrophil subsets that vary in their cytokine and chemokine production, macrophage activation and TLR expression (Tsuda, Takahashi et al. 2004) and in this way neutrophils may influence innate immune responses. However it is not clear if these subsets, broadly divided into pro-inflammatory and anti-inflammatory neutrophils, arise as a consequence of inflammation or whether they truly represent distinct lineages. Neutrophils may promote adaptive immune responses by providing an early source of IFN-γ in infection (Yin and Ferguson 2009) or by acting as antigen-presenting cells (Duffy, Perrin et al. 2012). Other work however, has highlighted the ability of neutrophils to suppress T cell function (Pillay, Kamp et al. 2012).

1.4 Cytokines and chemokines and their role in immune responses in the skin

Cytokines are small proteins that are produced by various cells in body and bind to specific receptors. They may act in an autocrine, paracrine and in

some circumstances, endocrine manner. Chemokines are chemoattractant cytokines that induce chemotaxis in nearby responsive cells promoting their recruitment along a gradient of increasing concentration towards the source cell. Chemokines are structurally divided into C, CC, CXC, CX3C chemokines bases on the distribution of cysteine motifs involved in the formation of disulphide bonds and have crucial roles in both homeostasis and disease states (Blanchet, Langer et al. 2012).

TLRs are expressed by antigen presenting cells such as macrophages and dendritic cells but are also present on a variety of other cell types in the skin such as keratinocytes, fibroblasts and endothelial cells (Miller and Modlin 2007). TLRs initiate downstream signalling events that culminate in the production of inflammatory cytokines, production of antiviral type 1 interferons, chemokines and antimicrobial peptides (Kawai and Akira 2011). Pro-inflammatory cytokines secreted early in the immune response predominantly by macrophages and dendritic cells include IL-1, TNF-α, IL-6 and IL-12 (Miller and Modlin 2007). TNF-α and IL-1 activate vascular endothelium to express adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 (Bevilacqua, Pober et al. 1987; Bertini, Sironi et al. 1992; Groves, Allen et al. 1995) augmenting recruitment of neutrophils, monocytes (Ming, Bersani et al. 1987) and skin-homing T cells (Picker, Kishimoto et al. 1991). IL-12 activates recruited NK cells to secrete large amounts of IFN-γ and both IL-12 and IFN-γ promote differentiation of $CD4^+$ T cells to into pro-inflammatory T_H1 cells (Trinchieri 1995). IFN-γ is also produced by activated T_H1 cells and activates macrophages to increase their antimicrobial activity and expression of MHC-II molecules (Basham and Merigan 1983; Nathan, Murray et al. 1983).

In response to stimulation by cytokines such as IL-1, TNF-α and IFN-γ, other resident cells such as keratinocytes, dermal fibroblasts and endothelial cells as well as inflammatory cells produce various chemokines (Elbe, Foster et al. 1996) which play a role in leukocyte recruitment. Members of the CXC chemokine subfamily such as CXCL8/IL-8 are important for neutrophil recruitment and are significantly up regulated in psoriatic skin accounting for

the characteristic intra-epidermal pustules that are seen (Hautz, Wolfram et al. 2012). Members of the CC chemokine subfamily such as CCL2/MCP-1 and CCL5/RANTES are thought to predominantly attract monocytes (Schroder 1995). Both CXC and CC chemokines attract T cells (O'Garra, McEvoy et al. 1998; Rossi and Zlotnik 2000). Therefore, PRRs activate signalling that induces an innate response and shapes the adaptive immune response according to the stimulating pathogen.

1.5 Impact of ageing on health

Over the last 30 years the number of centenarians in the UK has increased five fold from 2,500 in 1980 to 12,640 in 2010 (ONSa 2011) and in 2011 it was estimated that just under 1% of the total population was aged 90 and over (ONSb 2013). However, ageing is accompanied by a decline in effective immune responses resulting in increased susceptibility to infections and reduced responses to vaccines.

There is an increase prevalence of infections such as urinary tract infections, lower respiratory tract infections, skin and soft tissue infections, intra-abdominal infections, infective endocarditis, bacterial meningitis, tuberculosis and herpes zoster in the elderly, as well as increased morbidity and mortality from these infections (Yoshikawa 2000). Paradoxically ageing is also associated with a state of chronic low-level inflammation with an increase in circulating levels of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β referred to as inflamm-ageing (Fagiolo, Cossarizza et al. 1993; Franceschi, Capri et al. 2007). Inflamm-ageing is thought to contribute to a variety of age-related pathologies such as atherosclerosis (Vasto, Candore et al. 2007), type II diabetes, osteoporosis and cognitive decline (Cevenini, Monti et al. 2013).

Vaccines are one way to boost immune responses and in the US four are recommended for persons 60 years and over (CDC - 2013). However the response of old people to these vaccines is less robust than in younger groups due to age-related changes in the immune system (Weinberger,

Herndler-Brandstetter et al. 2008). Vaccine efficacy in the elderly to protect against influenza has been estimated to be between 17-53% compared to 70-90% in young adults (Goodwin, Viboud et al. 2006). The efficacy of the zoster vaccine to prevent herpes zoster also declines with age (64% in those aged 60-69 years, 38% in those >80 years) (Levin 2012).

Given the change in population demographic it is becoming increasingly important to understand age-related immune defects if we are to match the increased life expectancy with quality of life.

1.5.1 Effect of ageing on T cells

T cells develop in the thymus but there is a steady loss of functional thymic tissue with age that proceeds rapidly before middle age and relatively slowly thereafter and is almost complete by the sixth decade (Steinmann, Klaus et al. 1985; Aspinall and Andrew 2000). Despite this, the size of the T cell pool is maintained during adult life by turnover of pre-existing populations in response to homeostatic mechanisms (Surh and Sprent 2008). However, the composition switches from one of mostly naïve T cells in young individuals to one of predominantly memory T cells in later life, with an accumulation of end-differentiated effector cells particularly within the $CD8^+$ T cell subset (Goronzy and Weyand 2012). This increase in memory T cells may represent the cumulative effect of antigen encounters as well as homeostatic expansion.

However, one potential consequence of repeated cell division is shortening of telomeres in T cells with ageing (Rufer, Brummendorf et al. 1999; Fletcher, Vukmanovic-Stejic et al. 2005; Akbar and Vukmanovic-Stejic 2007). Telomeres are repeating hexameric sequences of DNA that protect the ends of chromosomes (Blackburn 2005). However, each cell division results in loss of approximately 50 base pairs of telomeric DNA due to the inability of DNA polymerase to fully replicate to the ends of the chromosomes during DNA replication (Harley, Futcher et al. 1990). The rate of telomere loss can be reduced by the enzyme telomerase that synthesises hexameric repeats

restoring telomere length (Blackburn 2005). However, although this enzyme is initially up regulated upon T cell activation, with repeated stimulation T cells lose the ability to induce telomerase (Maini, Soares et al. 1999; Plunkett, Soares et al. 2001; Valenzuela and Effros 2002). At a critical minimal telomere length, the cell enters cell cycle arrest and a state of replicative senescence (Weng, Levine et al. 1995; Effros and Pawelec 1997). CD28 co-stimulation plays a key role in up-regulating telomerase activity during activation and a decline in telomerase activity parallels the loss of CD28 expression (Weng, Levine et al. 1996; Valenzuela and Effros 2002), also seen in ageing.

Ageing is associated with changes in expression of surface molecules on T lymphocytes and the accumulation of $CD28^-$ memory T cells, predominantly within the $CD8^+$ T cell subset, is a consistent age-associated change in humans (Fagnoni, Vescovini et al. 1996). CD28 is a co-stimulatory molecule that interacts with CD80 and/or CD86 expressed on activated APCs and plays a role in T cell activation, proliferation and survival (Riley and June 2005). $CD28^-$ T cells show reduced proliferation in response to TCR stimulation (Azuma, Phillips et al. 1993). However $CD28^-$ T cells do possess enhanced cytotoxic capacities (Azuma, Phillips et al. 1993), consistent with their predominant effector-memory status.

Elderly individuals also have expanded end-differentiated effector T cells populations that re-express CD45RA, particularly within $CD8^+$ T cell subset (Akbar and Fletcher 2005). These end-differentiated cells are usually oligoclonal indicative of chronic antigen stimulation and can be seen in people with chronic cytomegalovirus (CMV) infection (Chidrawar, Khan et al. 2009). These populations may also in part be expanded in response to homeostatic cytokine induced proliferation (Chiu, Fann et al. 2006; Griffiths, Riddell et al. 2013). Such oligoclonal expansions of memory lymphocytes are proposed to be detrimental in ageing as they take up ‘immunological space’ depriving other T cells of resources such as cytokines and surface ligands, constraining

adaptive immune response to other existing or new antigens (Khan, Hislop et al. 2004).

Age-related functional changes are also evident in the naïve T cell compartments and naïve $CD4^+$ T cells from aged humans show reduced *in vitro* responsiveness to TCR and co-receptor stimulation and altered cytokine profile compared to naïve $CD4^+$ T cells from young individuals (Thompson, Wekstein et al. 1984; Linton, Haynes et al. 1996; Weng 2006)

1.5.2 Effect of ageing on innate immunity

Previous studies have shown that ageing appears to affect all the major cell types involved in the innate immune response. Most aspects of neutrophil function including chemotaxis, phagocytosis and generation of superoxide in response to stimulation by host and pathogen factors are decreased in the elderly (Panda, Arjona et al. 2009). In addition neutrophils are less able to respond to survival signals such as GM-CSF provided at sites of inflammation (Fortin, Larbi et al. 2007), reducing their ability to resolve infections.

A number of monocyte/macrophage functions decline with ageing. In particular monocytes from old individuals show an age-associated defect in TLR-induced production of TNF-α and IL-6, particularly in response TLR 1/2 stimulation and a generalised defect in TLR induced up-regulation of CD80 in monocytes compared to young individuals (van Duin, Allore et al. 2007; van Duin, Mohanty et al. 2007). In a study using West Nile virus (WNV), a mosquito borne infection with disproportionate morbidity and mortality in older individuals, it was shown that interaction between the WNV envelope and the macrophage surface molecule dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) *in vitro*, reduced expression of TLR3 in macrophages of young people but not in the old (Kong, Delroux et al. 2008). It was speculated that this persistence of TLR-3 might mediate an exaggerated inflammatory response and the more severe WNV-induced disease seen in elderly sufferers.

Healthy older people appear to have similar proportions of conventional DCs and pDCs in blood compared to young people (Jing, Shaheen et al. 2009). The number of Langerhans cells in the skin have been shown to be reduced in old skin (Grewe 2001). The effects of ageing on the inflammatory response by different DC subsets and subsequent priming of T cells is complex with most studies showing impaired responses, some showing preserved responses and a few enhanced responses (Wong and Goldstein 2013). There is general agreement that ageing impairs the IFN I response by pDCs to viral infection (Jing, Shaheen et al. 2009; Sridharan, Esposo et al. 2011).

1.5.3 Studying the effects of ageing on *in vivo* cutaneous DTH responses

Murine models have provided useful insights into immune mechanisms but there are limitations to their use for investigating human ageing processes and cutaneous DTH responses. Mice have considerably shorter lifespans yet have significantly longer telomeres suggesting telomere erosion, a characteristic finding of ageing in humans, is not as relevant in murine ageing (Kipling and Cooke 1990). Also, loss of CD28 expression on memory T cells seen in humans with subsequent accumulation of this subset with ageing does not occur in mice (Ortiz-Suarez and Miller 2002). DTH responses in mice are assessed by the degree of footpad or ear swelling whereas in humans the forearm is typically used. Site related variations in DTH responses in humans have been reported and would presumably also apply between species (Wammanda, Gambo et al. 2006). In mice the predominant T cell population in the skin resides in the epidermis and bears γ/δ TCRs whereas in humans T cells predominantly bear the α/β TCR and are found in the dermis (Elbe, Foster et al. 1996). Also higher concentrations of antigen are required to elicit a DTH response in mice compared to humans (Mestas and Hughes 2004). In addition there are differences in leukocyte transit times, polarisation of T cell populations and chemokines and chemokine

receptor expression between the two species (Mestas and Hughes 2004). Finally, VZV, the antigen of interest in this study, only produces clinically apparent infection in humans.

However, studies in humans can be limited by ethical constraints and the difficulty in extricating relevant findings from the impact of confounding factors. With this in mind, the SENIEUR protocol was designed in 1984 and defined strict admission criteria to immunologerontological studies to limit the influence of extrinsic factors such as disease and pharmacological interference (Ligthart, Corberand et al. 1984) allowing the study of 'healthy ageing'. However, study of this limited population using the SENIEUR protocol has suggested these elderly individuals have only modest changes in immune response but this does not reflect the circumstances of the majority of the aged population (Castle, Uyemura et al. 2001). Strict application of the SENIEUR protocol has shown to exclude 70% of community dwelling old people who consider themselves to be healthy (Wick and Grubeck-Loebenstein 1997) and as many as 90% of old people admitted to congregate housing (for those who are 'self-supporting, reasonably healthy old people') (Castle, Uyemura et al. 2001). Some studies have used an 'almost healthy' elderly population who would be excluded from the SENIUER protocol due to one or two minor criteria. Study of this 'almost healthy' elderly population does show significant differences in immune function compared to young individuals, not seen in the healthy old (Mysliwska, Bryl et al. 1999). A modified version of the SENIUER protocol was also used in this thesis with a view to exclude significant co-morbidity and pharmacological interference but select a representative rather than exceptional group of old individuals.

Previous *in vivo* human studies have shown that old individuals have a reduced ability to mount a cutaneous DTH response to recall antigens (Moesgaard, Lykkegaard Nielsen et al. 1987; Marrie, Johnson et al. 1988). Our group has shown that the reduced recall response to cutaneous PPD and *C. albicans* antigen seen in elderly humans does not reflect a global immune deficit since they show robust PBMC responses to antigen stimulation *in vitro*

(Agius, Lacy et al. 2009). We have also shown that in the cutaneous DTH response to *Candida albicans*, there is a decreased ability of macrophages in the skin of older humans to secrete inflammatory cytokines such as TNF-a following antigen challenge (Agius, Lacy et al. 2009). It is proposed that reduced pro-inflammatory cytokine secretion early in the DTH response to *C. albicans* fails to sufficiently activate dermal vessel endothelium to permit T cell entry and generate an adequate DTH response in the elderly.

1.6 Aims and Objectives

Ageing is associated with immune dysfunction. This is reflected in the increased frequency and severity of infectious and non-infectious diseases. Whilst many studies use murine models of disease or focus on circulating leukocytes populations in humans, we aim to study a memory T cell response *in vivo* in humans to a clinically relevant antigen in the appropriate organ, in this case, VZV antigen in the skin. By studying the immune response in the blood and skin of old people and comparing with it those in young people, we aim to gather pertinent data regarding the attenuation of cutaneous immunity during ageing in humans. This is becoming an increasingly important challenge given the changing population demographic.

AIM

To characterise the effects of ageing on the immune system in the skin in healthy old individuals.

OBJECTIVES

The objectives of this project were to:

1. Compare the cutaneous clinical response with peripheral blood responses to VZV antigen in the old and young.

2. Characterise and compare the kinetics and magnitude of the cellular response to intradermal injection of VZV antigen in the old and young.

3. Characterise the relationship between VZV-specific memory $CD4^+$ T cells and $CD4^+Foxp3^+$ Tregs that accumulate in response to the intradermal injection of VZV antigen.

Chapter 2. Material and methods

2.1 Volunteer recruitment

This work was conducted with approval from the Ethics Committee of the Royal Free Hospital. Healthy young individuals, aged 18 - 40 years (n=80, 43 females, 37 males, median age 27.5 years) and healthy old individuals, 65 years of age or above (n=36, 24 females, 12 males, median age 73.5 years), with a history of chickenpox were recruited into the study. Exclusion criteria for entry into the study were based on a modified version of the clinical criteria stated in the SENIEUR protocol (Ligthart, Corberand et al. 1984) (Table 2.1). Laboratory analyses were not undertaken and individuals on prescribed mediation were not excluded unless the medication was known to be immunosuppressive in nature. Whilst these latter deviations from the SENIEUR protocol may reduce the homogeneity of the group of elderly individuals studied, it allowed for more realistic recruitment into our study by increasing the number of potential volunteers, reducing the intrusive nature of the study by omitting initial screening blood tests, and reducing the number of visits involved for participants, whilst still being representative of a sample of the healthy ageing population. Written informed consent was taken from each participant and study procedures were performed in accordance with the principles of the declaration of Helsinki.

Unable to give written informed consent
Significant co-morbidity (renal impairment or failure, heart failure, diabetes)
Past history of neoplasia in the last 10 years (excluding basal cell carcinoma)
Previous treatment with chemotherapy or radiotherapy
Immunosuppressive medication (systemic steroids in the last month or any other systemic immunosuppressant within the last 6 months)
Recent infection or immunisation (within last month)
Pregnancy or breast feeding
Previous history of hypersensitivity to skin testing
Known immunodeficiency
History of chronic inflammatory disease

Table 2.1 Exclusion criteria

2.2 Skin testing

Volunteers were given an intradermal injection of 0.02ml of VZV antigen (The Research Foundation for Microbial Diseases of Osaka University, BIKEN) into the medial aspect of the proximal forearm to induce a delayed type hypersensitivity response. Correct administration into the intradermal compartment gave rise to a well-defined, short-lived, pale skin bleb at the site of injection. This site on the forearm was used as it is relatively protected from sun exposure and hence relatively spared from the immunosuppressive effects of acute UV exposure and photo-ageing related to chronic sun exposure. VZV antigen is prepared from VZV infected human diploid cells (Asano and Ahmed 1996; Takahashi, Okada et al. 2003). For some of the experiments, volunteers were also injected intra-dermally with normal saline (0.02ml) in the contralateral forearm to provide an internal control for the detection of trauma-related changes secondary to the intradermal injection. Unfortunately the carrier solution for the VZV antigen, TCM-199, which would have provided an ideal control, is not commercially available for intradermal use in humans. It contains amino aids and vitamins in addition to inorganic salts.

The response to the skin test antigen was measured typically between 48-72 hours after intradermal injection. The following parameters: (i) diameter of induration (ii) palpability (iii) change in erythema from baseline, were assessed. These were scored according to Table 2.2. The final clinical score was calculated from the sum of these individual scores.

Erythema was measured using a DermaSpectrometer (Cortex Technology, Hadsund, Denmark) (Figure 2.1B). This is a portable, handheld instrument and consists of a light emitting diode and a photodetector to measure absorbed and reflected light from the skin, providing a numerical measure of erythema, the erythema index (EI). The change in erythema index from baseline was calculated by subtracting the average of three readings taken from non-injected skin from the average of three readings taken over the injected site.

Clinical Score	**0**	**1**	**2**	**3**	**4**	**5**	**Score**
Change in Erythema-index (EI)	0	1-5	6-10	11-15	>16		
Size of Induration (mm)	0	1-5	6-10	11-15	16-20	>21	
Palpability	Nil	Just palpable	Easily	Marked	Very marked		
						Total	

Table 2.2 Clinical scoring system

2.3 Skin sampling

A skin suction blister was raised or a 5mm punch biopsy was taken from the injected site at a given time point between 0 and 7 days after skin testing.

2.3.1 Suction blisters

2.3.1.1 Suction blister induction

Skin suction at low pressure over a few hours has shown to induce a split between the epidermis and dermis at the level of the lamina lucida giving rise to a blister (Kiistala 1968) and the blister fluid contained is representative of skin interstitial fluid (Rossing and Worm 1981). To induce a skin suction blister, negative pressure of 25-40kPa (200-300mmHg below atmospheric pressure) was applied via a suction chamber (Medical Engineering, Royal Free Hospital, UK) positioned over the site of skin testing for 2-4 hours using a portable clinical suction pump (VP25, Eschmann, Lancing, UK) (Figure 2.1). Suction was applied until a unilocular blister measuring 10-15mm was formed. The blister was protected overnight with a rigid adhesive dressing assembled using a Comfeel plus ulcer dressing (Coloplast, Peterborough, UK), a universal top (Sterilin, Fisher Scientific UK Ltd, Loughborough, UK), Micropore tape (3M healthcare, Loughborough, UK) and Tubigrip bandaging (Seton Healthcare Group plc, Oldham, UK).

The following day, approximately 18-24 hours after induction, the blister fluid was aspirated using a sterile 21G needle and a 2ml syringe (Terumo Europe, Belgium). The time lag between blister induction and aspiration was to allow cells from the site of antigen challenge in the skin to accumulate in the blister fluid. The recorded time of sampling was from the time of VZV antigen injection up to the time of blister fluid aspiration. Blister fluid was suspended in 1.5ml conical tubes (Alpha Laboratories Ltd, Eastleigh, UK). The blistered site was sprayed with Betadine dry powder spray (Seton Healthcare Group plc, Oldham, UK) and the site dressed with a Cosmopor E dressing (Hartmann, Heywood UK). Volunteers were advised to leave the dressing in place and to keep it dry for 48 hours before removing and leaving the wound open to the air. The suction cups were dismantled after use and disinfected in Barrycidal 36 (Heraeus Instruments Ltd, Brentwood, Essex, UK) for a minimum of 24 hours.

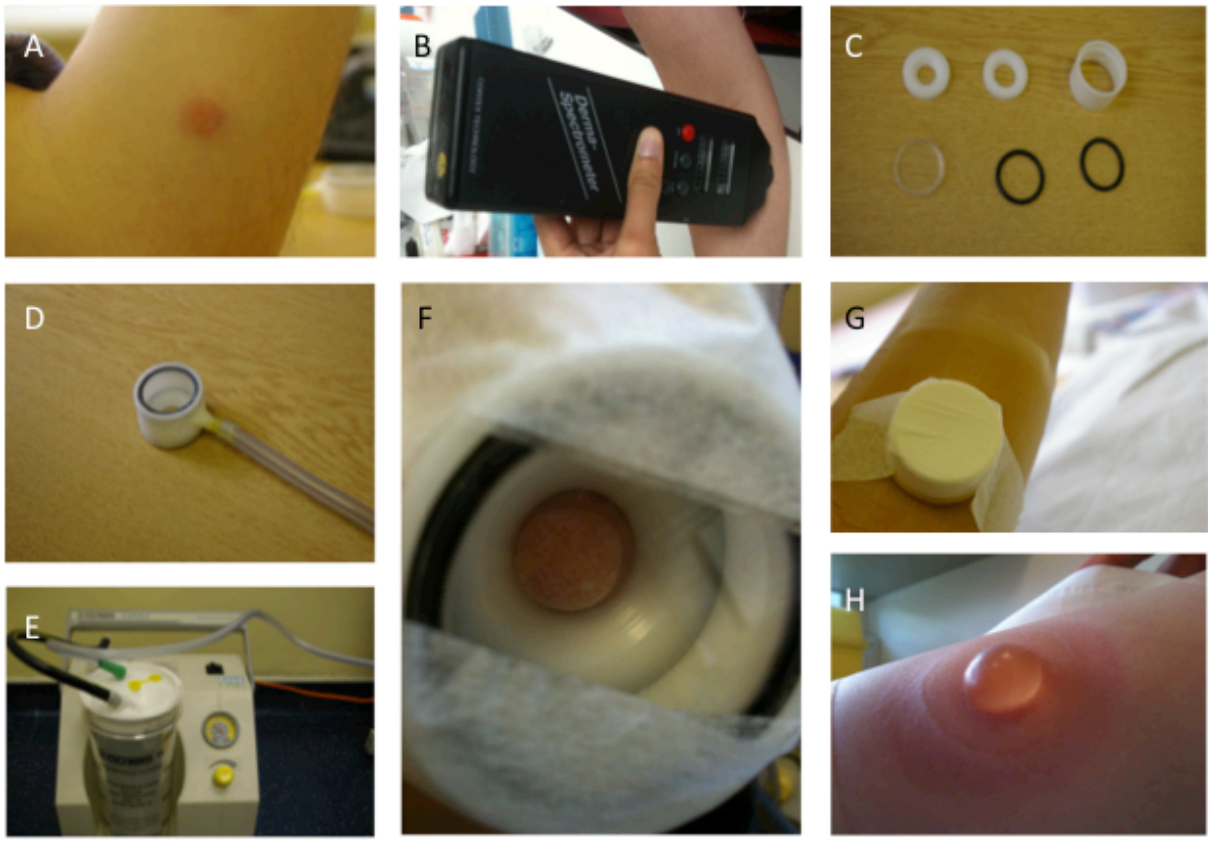


Figure 2.1: The clinical DTH response in the skin and skin suction blister Induction

(A) DTH responses were induced by intradermal injection of VZV antigen in volunteers with a history of chickenpox. Clinical responses were assessed 48-72 hours after injection by palpability, induration and erythema. (B) The erythema index was measured using a DermaSpectrometer to provide objective values. (C,D) Suction chambers were made up of a nylon cup, a transparent Perspex lid and a template. The size of the template used was adjusted according to the size of the clinical response. Rubber O-rings were used between the components to allow an airtight seal to be formed when the chamber is assembled. (E,F) Suction chambers were connected to a portable clinical suction pump via sterile disposable tubing and positioned securely over the injected skin site. (F) Suction blisters were induced by applying negative pressure (25-40kPa (200-300mmHg) over 2-4 hours. (G) The formed blister was protected overnight using a rigid universal container top placed over a 5x5cm Comfeel dressing, secured with micropore tape. (H) The blister was aspirated 18-24 hours after formation using a sterile 21G needle and a 2ml syringe.

2.3.1.2 Suction blister cell isolation

Blister fluid was microcentrifuged at 3000 rpm for 4 minutes (Heraeus Biofuge Pico) to pellet the cellular contents. The supernatant was removed and the cell pellet was re-suspended in 500μl of RPMI 1640 containing 10% human AB serum, 100U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine (all from Sigma Aldrich, Gillingham, Dorset, UK).

2.3.1.3 Counting of blister cells

Blister mononuclear cell numbers were counted using a haemocytometer. Tryptan blue was used to assess cellular viability. A 10μl aliquot of blister cell suspension was mixed 1:1 with Trypan blue (Sigma-Aldrich, Gillingham, Dorset, UK) and viable non-stained cells were counted.

2.3.2 Skin biopsy

2.3.2.1 Biopsy procedure

A 5mm punch biopsy (Steifel, Sligo, Ireland) was taken of the centre of the injected skin test site or of normal skin. Prior to biopsy the area was infiltrated with 1% lidocaine hydrochloride local anaesthetic (Hameln Pharmaceuticals Ltd, Gloucester, UK). Biopsy wounds were closed with a 4.0 Ethilon polyamide non-absorbable suture (Johnson & Johnson Intl, Belgium) and the site dressed with a Cosmopor E dressing (Hartmann, Heywood, UK). Volunteers were advised to leave the dressing in place and keep it dry for 24 hours before removing and leaving the wound open to air. The suture was removed after 7 days. Skin biopsies were transported to the laboratory in sterile gauze soaked in saline within 15 minutes of obtaining it to prevent desiccation of the skin sample.

2.3.2.2 Biopsy storage and sectioning

Biopsies were mounted in OCT (optimal cutting temperature compound; Bright Instrument Company Ltd, Huntingdon, UK) on cork disks, orientated with the epidermis perpendicular to the cork disk, and snap frozen in isopentane (Sigma-Aldrich, Gillingham, Dorset, UK) cooled in liquid nitrogen. The samples were stored in a freezer at -80°C. 6μm frozen sections were cut at -20°C using a Bright 5040 microtome (Bright Instrument Company Ltd., Huntingdon, UK) on to poly-L-lysine coated glass slides (Sigma-Aldrich, Gillingham, Dorset, UK). Poly-L-lysine coated slides were used to help the skin section adhere to the slide. Two sections were mounted on to each slide. The sections were left overnight to air-dry and then fixed in fresh acetone for 10 minutes, followed by 99% ethanol for 10 minutes. The sections were air-dried for a further 10 minutes and then wrapped individually in cling-film and stored in a freezer at -80°C.

2.4 Blood sampling and PBMC isolation

Heparinised blood was collected from volunteers prior to VZV skin test antigen injection or at the time of blister aspiration. Heparinised blood was mixed with an equal volume of Hanks Balanced Salt Solution (HBSS) (Sigma Aldrich, Gillingham, Dorset, UK) and layered on to Ficoll-Paque (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK) in 50ml Falcon tubes. This was centrifuged for 20 minutes at 2000rpm with no brake. The buffy coat at the interphase layer was retrieved and washed in excess HBSS twice, with centrifugation at 1800rpm and 1200rpm for 10 minutes after the first and second wash respectively. The cell pellet was then re-suspended in complete medium (RPMI 1640 containing 10% human AB serum, 100U/ml penicillin, 100μg/ml streptomycin and 2mM L-glutamine (all from Sigma Aldrich, Gillingham, Dorset, UK)). Typically, 0.5-2 $x10^6$ cells were isolated per 1ml of venous blood.

2.5 Immunohistology

2.5.1 Antibodies used in the study

Antigen	Source	Clone[a]	Isotype	Dilution	Amplification/Detection[b]
INDIRECT IMMUNOFLUORESCENCE					
CD4 Biotin	BD 347321	SK3	IgG1, κ	1:50	Streptavidin-Cy3 1:100
CD4 Leu-3a	BD 346320	SK3	IgG1	1:10	Donkey anti-mouse IgG AlexaFlour488 1:200
CD8 biotin	BD 555365	RPA-T8	IgG1, κ	1:50	Streptavidin-Cy3 1:100
Foxp3 biotin	E-biosciences 13-4776-82	PCH101	Rat IgG2a, κ	1:100 o/n	Streptavidin-Cy3 1:100
CD62E biotin	Abcam 36688	ENA1	IgG1	1:50	Streptavidin-Cy3 1:100
CD163	Acris	5C6-FAT	IgG1	1:100	Goat anti-mouse IgG1 AlexaFluor568, 1:250
CD11c	BD 550375	B-ly6	IgG1	1:100	Goat anti-mouse IgG1 AlexaFluor568, 1:250 AlexaFluor488, 1:250
BDCA-1	Miltenyi	AD5-8E7	IgG2a	1:100	Goat anti mouse IgG2a AlexaFluor568, 1:250
TNF-α FITC	BD	6401.1111	IgG	1:10	Goat IgG A488 anti-fluorescein/Oregon, 1:200
DIRECT IMMUNOFLUORESCENCE					
Ki67 FITC	BD 556026	B56	IgG1, κ	1:50	
CD31 FITC	BD 555445	WM59	IgG1, κ	1:50	

Table 2.3 Antibodies used for Immunofluorescence

o/n = overnight

[a] = all are murine monoclonal antibodies unless stated

[b] = all detection antibodies are from Invitrogen with the exception of Streptavidin-Cy3 (Cedarlane labs Ltd. CLCSA1010)

Antigen	Source	Clone[a]	Isotype	Dilution	Detection, developing agent
Neutrophil elastase	Dako	NP57	IgG1, κ	1:200	Biotinylated horse anti-mouse antibody; avidin-biotinylated enzyme complex (VECTASTAIN ABC, Vector Laboratories); AEC
BDCA-2	Miltenyi	AC144	IgG1	1:10	
CD4	BD 340133	SK3	IgG1, κ	1:100	
BDCA-3	Miltenyi	AD5-14H12	IgG1	1:20	
DC-LAMP	Beckman Coulter	104.G4	IgG1	1:50	
CD11c	BD	B-ly6	IgG1, κ	1:100	
CD163	Acris	5C6-FAT	IgG1	1:100	

Table 2.4 Antibodies used for Indirect Immunohistochemistry

[a] = all are murine monoclonal antibodies unless stated

AEC = 3-amino-9-ethyl carbazole

2.5.2 Indirect Immunofluorescence

In this technique a primary unconjugated antibody is used to detect the antigen of interest in a tissue section. A secondary fluorescently labelled antibody, raised in a different animal host and specific for the animal and immunoglobulin class of the primary antibody is then added and allowed to bind the primary antibody. The fluorescent signal, which is representative of the presence of the antigen of interest, can then be visualised using a fluorescence microscope. Multiple secondary antibodies may bind the primary antibody and in this way also amplifies the signal generated. The primary and secondary antibodies used in this study are shown in table 2.3. In some cases, a directly conjugated primary antibody was used e.g., CD31 FITC, Ki67 FITC, and these signals did not require an amplification step i.e. direct immunofluorescence. For TNF-α FITC, a secondary anti-FITC antibody was used to amplify the signal. Where the simultaneous presence of two antigens in a single tissue section is being investigated, it is preferable for the

primary antibodies to be raised in different animal hosts to reduce the risk of the secondary antibodies binding to both primary antibodies.

Slides mounted with 6um skin sections as described above and stored at -80^{0}C were placed in a moist staining box at room temperature for 15minutes before being unwrapped from their cling film cover. Each section was then circled with a Dako pen (Dakocytomation, Glostrup, Denmark) that provides a water repellent barrier preventing escape of solutions applied to the sections. A drop of freshly prepared 1xPBS(Sigma Aldrich) (dissolve 2 PBS tablets in 400ml sterile water) was added to each ringed section to prevent drying of the tissue. Slides were placed in a slide rack and washed in 1xPBS in a glass container for 5 minutes. Excess PBS was removed by tapping the slide and a drop of Dako non-serum protein block (Dakocytomation, Glostrup, Denmark) was added to the ringed sections for 20 minutes to reduce non-specific antibody binding. The blocking agent was removed by tapping the slide and the primary antibody, diluted in 1xPBS at a predetermined dilution, was added to each section (50µl/section). Slides were left to incubate at room temperature in the dark for 1 hour. For Foxp3 staining the primary antibody was left to incubate overnight as a prolonged incubation period results in better staining with this antibody.

At the end of the incubation period with the primary antibody, slides were washed in 1x PBS for 5 minutes in the dark, twice. Excess PBS was removed by tapping the slide and secondary antibody diluted in 1xPBS (50µl/section) was added to each section and left to incubate at room temperature in the dark for 45 minutes. Next, slides were washed for 10 minutes in 1xPBS in the dark, twice. Filter paper was used to blot any remaining PBS from the slide and sections were mounted with Vectashield containing DAPI (Vector Laboratories Ltd, Peterborough, UK) and covered with a 22x22mm square coverslip. Vesctashield mounting media remains a viscous liquid on the slide preventing the sections from drying out and reduces photo-bleaching. The coverslips were secured with clear nail varnish and the slides were placed in a slide holder and stored in the dark at -20^{0}C.

Staining for co-expression of TNF-α with either CD163 or CD11c, and CD11c expression with BDCA-1 was performed by Dr M. Vukmanovic-Stejic and Dr J. Duculan-Fuentes in Professor J. Krueger's laboratory at the Rockefeller University in New York using a slightly different protocol. Skin sections were blocked in 10% normal goat serum (Vector Laboratories Ltd) for 30 minutes. Primary antibody to CD163 or CD11c was diluted in 10% goat serum and sections were incubated overnight at 4^0C. A secondary goat anti-mouse IgG1 antibody conjugated with Alexa Fluor 568 was added followed by overnight incubation with directly conjugated TNF-α FITC and amplified with goat anti-FITC Alexa Fluor 488.

2.5.2.1 Biotin-Streptavidin Cy3 technique

Some of the primary antibodies used were biotinylated e.g. Foxp3, CD8 and CD4. The detection and amplification step in these cases was performed using streptavidin protein conjugated to the fluorochrome. Biotin binds streptavidin with a high affinity and specificity. The protocol for their use in indirect immunofluorescence was exactly the same as described above.

2.5.2.2 Control slides

Control slides were used to distinguish between specific antigen binding and that due to background autofluorescence e.g. from the collagen fibres in the dermis as well as any non-specific binding of the secondary antibodies. Tissue sections known to express the antigen(s) of interest i.e. positive controls, tissue sections where the antigen of interest is expected to be absent i.e. negative controls, tissue sections stained with just the secondary antibody to detect non-specific binding as well as isotype controls were used as needed, to allow correct determination of the fluorescent signal.

2.5.2.3 Quantification of Immunofluorescence

Images were acquired using appropriate filters of a Leica DMLB microscope with Leica N PLAN 20 or 40 objective and a Cool SNAP-Pro cf Monochrome Media Cybernetics camera, controlled by Image-Pro PLUS 6.2 software.

When counting the numbers of cells in perivascular infiltrates (PVs), the five largest PVs present in the upper and mid-dermis of each section were counted. Cell numbers were expressed as the mean absolute cell number per PV. In normal skin, the five largest accumulates present in the upper and mid-dermis of each section were counted. When assessing the expression of E-selectin on CD31 expressing endothelial cells, the entire upper and mid-dermis of the section was examined. Slides were assessed by one of two researchers and reviewed by both if there were any uncertainties.

2.5.3 Indirect Immunohistochemistry

The biotin-avidin technique that employs the extremely strong avidin – biotin interaction was used. This technique uses a primary unconjugated antibody to detect the antigen of interest in the tissue section. A secondary biotinylated antibody raised in another animal host and specific for the animal and immunoglobulin class of the primary antibody binds the primary antibody. Avidin conjugated to a biotinylated horseradish peroxidase (avidin and biotinylated horseradish peroxidase macromolecular complex (ABC)) binds the biotinylated secondary antibody. Finally the chromogen 3-amino-9-ethyl carbazole (AEC), a peroxidase substrate is added and gives rise to a red/brown colour where it encounters ABCs and hence the brown colour seen under a light microscope is representative of the presence of the antigen of interest. Table 2.4 lists the primary antibodies used in this study.

Slides mounted with 6um skin sections as described above and stored at -80^0C were placed in a moist staining box at room temperature for 15 minutes before being unwrapped from their cling film cover. Slides were washed in 1xPBS for 5 minutes. Excess PBS was removed by tapping the slides and

sections were incubated with 50µl of 10% normal horse serum (prepared from the same species in which the secondary antibody is made) for 20 minutes. The slides were tapped again to remove the blocking serum before adding 50µl of the primary antibody diluted in 1% normal horse serum to each section. Sections were left to incubate overnight at 4^0C. Slides were rinsed by brief immersion in 1xPBS twice to remove unbound primary antibody before being washed in 1xPBS for 5 minutes. At this stage, ABC is prepared from a commercially available kit, Vectastain Elite ABC kit (Vector laboratories) by allowing 10µl of agent "A" (Avidin DH) and 10µl of agent "B" (biotinylated enzyme) to react in 1000µl of 1% normal horse serum diluted in PBS. The substrate AEC is prepared by dissolving 120mg of AEC in 15ml of N, N-dimethyl formamide (Sigma Aldrich). 0.1M acetate buffer is prepared by adding 79ml of 0.1M sodium acetate (13.61 g/L distilled H_20 and 21ml of 0.1M Acetic acid). The mixture is filtered through a 0.2 micron filter with a syringe and 2.5µl of 30% H202 is added to activate the reagent. AEC substrate was prepared in advance by Dr. J. Fuentes-Duculan in Professor J. Krueger's laboratory at the Rockefeller University in New York when skin sections were stained in their laboratory. Slides were tapped to remove PBS and sections were each incubated with 50µl of horse anti-mouse biotinylated secondary antibody diluted in 1% normal horse serum (1:200) for 30 minutes at room temperature. Slides were rinsed again by immersing twice in 1xPBS and then placed in 0.3% H_2O_2 (1:10 dilution of 3% Henry Schein Hydrogen peroxide solution in distilled water) for 15 minutes to ensure quenching of endogenous peroxidase. Slides were rinsed by immersing in 1xPBS twice and then washed in 1xPBS for 5 minutes. Excess PBS was removed by tapping slides and sections were incubated with VECTASTAIN ABC for 30 minutes at room temperature (50µl per section). The slides were rinsed and washed in PBS again before the AEC reagent was applied. Sections were closely monitored by repeated examination under a light microscope until the desired staining intensity developed. The slides were rinsed several times in distilled water and left to dry. The dry sections were mounted in aqueous mounting medium and covered with a square 22mm x 22mm coverslip.

2.5.3.1 Quantification of Immunohistochemistry

Slides were visualised using a light microscope (Nikon Eclipse E600) and a digital camera (Nikon DXM1200F camera with Eclipse Net software). In all cases, a field with a pre-determined area (1x1.2mm) was centred on the digital image with the epidermis at the top of the field, and the number of cells within it were counted manually using computer assisted image analysis software (Image J) (NIH Image 6.1;http://rsb.info.nih.gov/nih-image). Where more than a single frame was needed to examine the upper and mid dermis of the entire section, the average of the counts per field was used for analysis. Slides were assessed by one of two researchers and reviewed by both if there were any uncertainties.

2.6 Flow cytometry

Flow cytometry was used to analyse stained PMBCs and blister cells and was performed on BD LSR Fortessa or BD LSR II (both Becton Dickinson, NJ, USA) flow cytometers which have 4 and 5 lasers respectively, using FACS Diva software. FlowJo software (TreeStar, Inc) was used to further analyse the flow cytometer read out. Forward and side scatter profiles were used to identify the lymphocyte population. Dead cells and debris were excluded and the cells of interest further analysed according to the presence of cell markers. Single colour fluorochrome controls were used in each experiment to allow compensation of overlapping emission spectra from the different fluorochromes prior to each sample acquisition. Isotype controls were used to delineate positive populations where positive and negative populations were not clearly distinguishable. The antibodies and the concentration at which they were used for flow cytometry are shown in Table 2.5.

2.6.1 Surface staining by direct immunofluorescence

Monoclonal antibodies directly conjugated to a specific fluorochrome were

used to detect the expression of cell surface markers. 100µl aliquots of PBMC or blister cell suspension were placed in 5ml FACS tubes (Falcon, Becton Dickinson, NJ, USA). Surface antibodies were added and the samples incubated in the dark at 4^0C for 30 minutes. Samples were washed with 2ml PBS and centrifuged at 1800rpm for 5minutes. The supernatant was decanted and the cells re-suspended in 200µl of 0.5% paraformaldehyde (Sigma-Aldrich, Gillingham, Dorset, UK) in PBS. Samples were stored at 4^0C in the dark until run on the flow cytometer.

2.6.1.1 Detection of antigen-specific T cells

A MHC class II tetramer, HLA-DRB1*1501 iTAg MHCII tetramer (Beckman Coulter) complexed to VZV IE63 peptide 24 (amino acids 229-243, QRAIERYAGAETAEY), was used to detect VZV specific cells in samples taken from volunteers known to be HLA-DRB1*15 positive. CLIP peptide (PVSKMRMATPLLMQA) was used as a control. In these experiments, 100µl aliquots of PBMC containing $1x10^6$ cells and100µl blister cell suspension containing between approximately 20,000 – 300,000 cells were incubated with 2µl/ml tetramer at 37^0C in a humidified 5% CO_2 atmosphere for 1-2 hours in the dark, washed with 2ml PBS and centrifuged at 1500rpm for 5 minutes prior to addition of other surface antibodies. Subsequent washes were also followed by centrifugation at 1500rpm rather than 1800rpm in samples containing tetramer.

2.6.2 Intracellular staining

Intracellular staining was used to detect cytokine production by antigen specific cells after *in vitro* stimulation, proliferating cells by their expression of Ki67, and potential Tregs expressing Foxp3. In order to stain for cytokines, PBMCs and blister cells were fixed and permeabilied (Fix & Perm ® Cell Permeabilisation Kit; Caltag Laboratories) to allow antibodies to access the cytoplasm whilst maintaining the morphological scatter characteristics of the

cells. If a combination of both surface and intracellular antigen staining was required, cells were first incubated with surface antibodies as detailed above, washed with PBSA and centrifuged at 1800rpm for 5 minutes. The supernatant was decanted and the cell pellet re-suspended in 100µl of reagent A (Fixation medium containing formaldehyde) and left to incubate at room temperature for 15 minutes in the dark. Samples were washed with PBS and centrifuged for 5 minutes at 1800rpm. The supernatant was decanted and the cells re-suspended with 100µl of reagent B (Permeabilisation medium) and directly conjugated antibodies to the intracellular proteins of interest were added. Samples were left to incubate at 4^0C in the dark for 30 minutes. The cells were finally washed with PBSA (1% w/v bovine serum albumin and 0.02% sodium azide (both Sigma-Aldrich, Gillingham, Dorset, UK), centrifuged at 1800rpm for 5 minutes, the supernatant was decanted, and the cells re-suspended and fixed in 200µl of 2% paraformaldehyde in PBS. Samples were stored in the dark at 4^0C until they were analysed on the flow cytometer.

When samples were stained for Foxp3 or Ki67 expression, the Foxp3 Staining Buffer Set (Miltenyi Biotec) was used according to the manufacturer's instructions instead for fixation and permeabilisation. Briefly, after incubation with surface antibodies, samples were washed in PBS, centrifuged and the supernantant decanted as described above. Cells were re-suspended in1ml of freshly prepared fixation-permeabilisation solution (Fixation medium containing formaldehyde) and were left to incubate in the dark at 4^0C for 30minutes. 1ml of freshly prepared permeabilisation buffer solution was then added, and samples centrifuged at 1800rpm for 5minutes. The supernatant was decanted and this wash step repeated. Directly conjugated antibodies to the intracelluar antigens of interest were added and left to incubate for 30minutes at 4^0C in the dark. Samples were washed once more with 1ml of permeabilisation buffer, centrifuged at 1800rpm for 5 minutes, the supernatant decanted and the cells fixed in 200µl of 2% paraformaldehyde as described above. In samples where both surface staining with the tetramer agent and intracellular staining for Foxp3 expression was required, all centrifugation

occurred at 1500 rpm when the tetramer was added and 1700rpm once the antibody to FoxP3 was added. These variations in centrifugation speeds were a compromise between the optimum required for each of the agents. In cases where cytokines and either KI67 or Foxp3 were stained for together, the Foxp3 staining protocol was used.

Antigen	**Clone**[a]	**Isotype**	**Dilution**	**Source**
Ki67 FITC	B56	mIgG1,κ	1:10	BD 556026
CD3 APC	UCHT1	mIgG1,κ	1:15	BD 555335
CD3 ECD	UCHT1	mIgG1	1:100	Beckman Coulter A07748
CD4 PECy-7	SK3	mIgG1,κ	1:20	BD 557852
CD4 APC-H7	RPA-T4	mIgG1,κ	1:20	BD 560158
CD4 Alexa Fluor® 700	RPA-T4	mIgG1,κ	1:50	BD 557922
CD4 PerCP	SK3	mIgG1,κ	1:10	BD 345770
CD8 PerCP	SK1	mIgG1,κ	1:10	BD 345774
CD8 APC	RPA-T8	mIgG1,κ	1:10	BD 555369
CD8 FITC	SK1	mIgG1	1:10	BD 345772
CD45RA FITC	HI100	mIgG2b,κ	1:10	BD 555488
CD45RA Alexa Fluor® 700	HI100	mIgG2b,κ	1:20	BD 560673

CD45RA PE-Cy7	L48	mIgG1	1:25	BD 337186
CD27 APC-H7	M-T271	mIgG1,κ	1:20	BD 560222
CD28 FITC	CD28.2	mIgG1,κ	1:10	BD 555728
CD28 PE	CD28.2	mIgG1,κ	1:12.5	BD 555729
CLIP PE	N/A	N/A	1:50	Beckman Coulter
Tetramer PE	N/A	N/A	1:50	Beckman Coulter
CD127 Alexa Fluor® 647	HIL-7R-M21	mIgG1,κ	1:10	BD 558598
CD127 V450	HIL-7R-M21	mIgG1,κ	1:20	BD 560823
CD127 PE	HIL-7R-M21	mIgG1,κ	1:25	BD 557938
CD25 PE-Cy7	M-A251	mIgG1,κ	1:20	BD 557741
CD39 FITC	A1	mIgG1	1:33	Abcam 30423
Foxp3 Pacific Blue	206D	mIgG1,κ	1:20	Biolegend 320116
Foxp3 APC	3G3	mIgG1	1:10	Miltenyi
Foxp3-APC	PCH101	mIgG1,	1:10	eBioscience
CD14 PerCP	MϕP9	mIgG2b,κ	1:10	BD 345786
CD19 PerCP	4G7	mIgG1,κ	1:10	BD 345778

IFNγ V450	B27	mIgG1,κ	1:200	560371
IFNγ APC	B27	mIgG1,κ	1:200	554702
IL-2 PE	JES6-5H4	Rat IgG2b	1:10	Miltenyi
IL-2 FITC	5344.111	mIgG1	1:10	BD 340448
IL-10 Alexa Fluor® 488	JES3-9D7	Rat IgG1,κ	1:20	Biolegend 501411
IL-17 Alexa Fluor® 700	N49-653	mIgG1,κ	1:20	BD 560613
Viaprobe - Cell Viability Solution	N/A	N/A	1:20	BD 555816
LIVE/DEAD – Cell Viability Kit	N/A	N/A	1:200	Invitrogen 889457

Table 2.5 Antibodies used for flow cytometry

2.7 *In Vitro* Cell Culture

The following were used to identify functional VZV-specific cells:

2.7.1 Overnight Stimulation Assay

Antigen-specific $CD4^+$ T cells were identified using flow cytometry to detect cells containing IL-2 or IFN-γ after short term incubation with VZV lysate in the presence of brefeldin A. Brefeldin A enhances staining of intracellular cytokines by preventing their secretion from the cell by interfering with intracellular transporting mechanisms. PBMCs ($1x10^6$) or blister cell

suspension were incubated in sterile FACS polypropylene tubes (Elkay Laboratory Products, Hampshie, UK) with VZV lysate (Virusys) at a final concentration of 40µl/ml for 15 hours at 37^0C in a humidified 5% CO_2 atmosphere. After 2 hours of incubation Brefeldin A (Sigma-Aldrich, Gillingham, Dorset, UK) was added at a final concentration of 5µg/ml. A non-stimulated control and a positive control, the superantigen Staphylococcal exotoxin B added at a final concentration of 1µl/ml (Sigma-Aldrich, Gillingham, Dorset, UK), were also included. At the end of the incubation period, the samples were centrifuged at 1800rpm for 5 minutes. The supernatant was decanted and the cells re-suspended in PBS. Staining for intracellular cytokines was performed according to the protocol described above.

2.7.2 Ki67 Proliferation Assay

Antigen-specific $CD4^+$ T cells were identified using flow cytometry to detect cells expressing Ki67, a cellular marker for proliferation, after incubation with VZV lysate. Ki67 is present during all active phases of the cell cycle but is absent from resting cells. PBMCs ($0.5x10^6$ cells) suspended in complete medium were incubated with VZV lysate (Virusys) at a final concentration of 4µl/ml in a 48 well plate (Falcon, Becton Dickinson Labware). Samples were incubated at 37^0C in a humidified 5% CO_2 atmosphere for 3 days. A non-stimulated negative control was included. At the end of the incubation period, the contents of the wells were centrifuged at 1800rpm for 5 minutes and the supernatant decanted. Cells were washed by re-suspending in PBS, centrifuging again at 1800rpm for 5 minutes and were then finally re-suspended in PBS. Staining for Ki67, an intracellular protein, was performed according to the protocol described previously.

2.7.3 [3H] Thymidine Incorporation Assay

In this assay, radiolabelled thymidine is incorporated into new strands of DNA synthesized during cell division. The radioactivity from the sample is measured using a scintillation beta-counter and reflects the degree of cell

division that has occurred. VZV-specific $CD4^+$ T cells would be expected to proliferate in response to incubation with VZV lysate. 200µl aliquots of PBMCs suspended in complete medium at a concentration of $1x10^6$ cells/ml were added to a 96 well round bottomed tissue culture plate (Falcon, Becton Dickinson). VZV lysate (Virusys) was added to give a final concentration range of between 0-10µl/ml. Samples at each concentration were set up in triplicate. The cells were incubated for 6 days at 37^0C in a humidified 5% CO_2 atmosphere before adding 10µl of 0.0025MBq [3H] thymidine (Amersham Biosciences UK Ltd, Chalfont St. Giles, UK) to each well and incubating for a further 16 hours. Plates were stored at -20^0C until a time when they could be harvested onto glass fibre filter strips using a cell harvester (Wallac 1450 MicroBeta Trilux Liquid Scintillation & Luminescence Counter). Counts per million (cpm) from incorporated [3H] thymidine were determined by liquid scintillation counting and the mean value of triplicate wells used for data analysis.

2.8 Statistics

Statistical analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, California, USA). Non-parametric tests were predominantly used as the data could not be assumed to be normally distributed. The Mann-Whitney U test was used when comparing only two unpaired groups, and the Kruskall-Wallis test for comparing three or more unpaired groups. The Wilcoxon matched pairs test or a paired t test was used when comparing two groups of matched data. Linear regression analysis was used to assess correlation between clinical score and both the number of infiltrating perivascular T cells and proportion of Tregs.

Chapter 3: Cutaneous clinical and peripheral blood responses to VZV antigen in old and young individuals

3. 1 Introduction

Varicella zoster virus is an exclusive human herpes virus that infects most individuals in childhood in European countries (Bonanni, Breuer et al. 2009) causing chickenpox. Following resolution of the acute illness, VZV becomes latent and persists life-long in neurons. Reactivation of latent VZV causes herpes zoster (HZ), more commonly referred to as shingles, characterized by pain and a rash typically limited to a single dermatome, corresponding to the sensory ganglion in which latent VZV reactivated. Post herpetic neuralgia, where pain persists in an area previously affected by HZ is the major complication of HZ. It is established that adequate VZV-CMI is necessary to prevent HZ, although the mechanism of prevention is not known. VZV-specific T cells may interact directly with neurons to prevent VZV reactivation, although VZV-specific T cells, in contrast to herpes simplex virus, have not been detected in human sensory ganglia (Verjans, Hintzen et al. 2007). Alternatively VZV-specific T cells may respond rapidly in the event of reactivation limiting the infection before it can become clinically apparent (Quinlivan, Ayres et al. 2007; Cohrs, Mehta et al. 2008; Malavige, Jones et al. 2008). Both periodic asymptomatic reactivation and external exposure to VZV e.g. contact with children with chickenpox, may potentially boost VZV-specific T cells. Despite this the incidence of HZ increases with age from between 5-6.5 per 1000 individuals at age 60 years to 8-11 per 1000 individuals at age 70 (Donahue, Choo et al. 1995) in keeping with the age-related decline in VZV cell mediated immunity (Sadaoka, Okamoto et al. 2008).

A skin test containing VZV antigen has been used to determine CMI to VZV and skin responses decline with increasing age (Burke, Steele et al. 1982; Sadaoka, Okamoto et al. 2008). The clinical response to the VZV skin test has been shown to correlate with the VZV-specific CMI measured by the

IFN-γ Enzyme-Linked Immunospot Assay (ELISPOT) (Sadaoka, Okamoto et al. 2008). In this chapter we compare clinical responses to this VZV skin test between old and young volunteers and see how this relates to peripheral blood responses to VZV antigen.

3.2 Clinical response to VZV skin test

61 young (40 years) and 29 old (>65 years) individuals were tested with 0.02ml of VZV antigen solution injected intradermally into the inner aspect of the proximal forearm. The clinical response to the skin test was assessed between 48 and 72 hours after injection, for erythema, diameter of induration and ease of palpability, and was assigned a clinical score based on these parameters (Table 2.2). The sum of the clinical scores in each age group was used to calculate a mean score for both groups. The clinical response to VZV skin test antigen was significantly diminished in old individuals compared to young (***p=<0.0001, Mann Whitney test) (Figure 3.1).

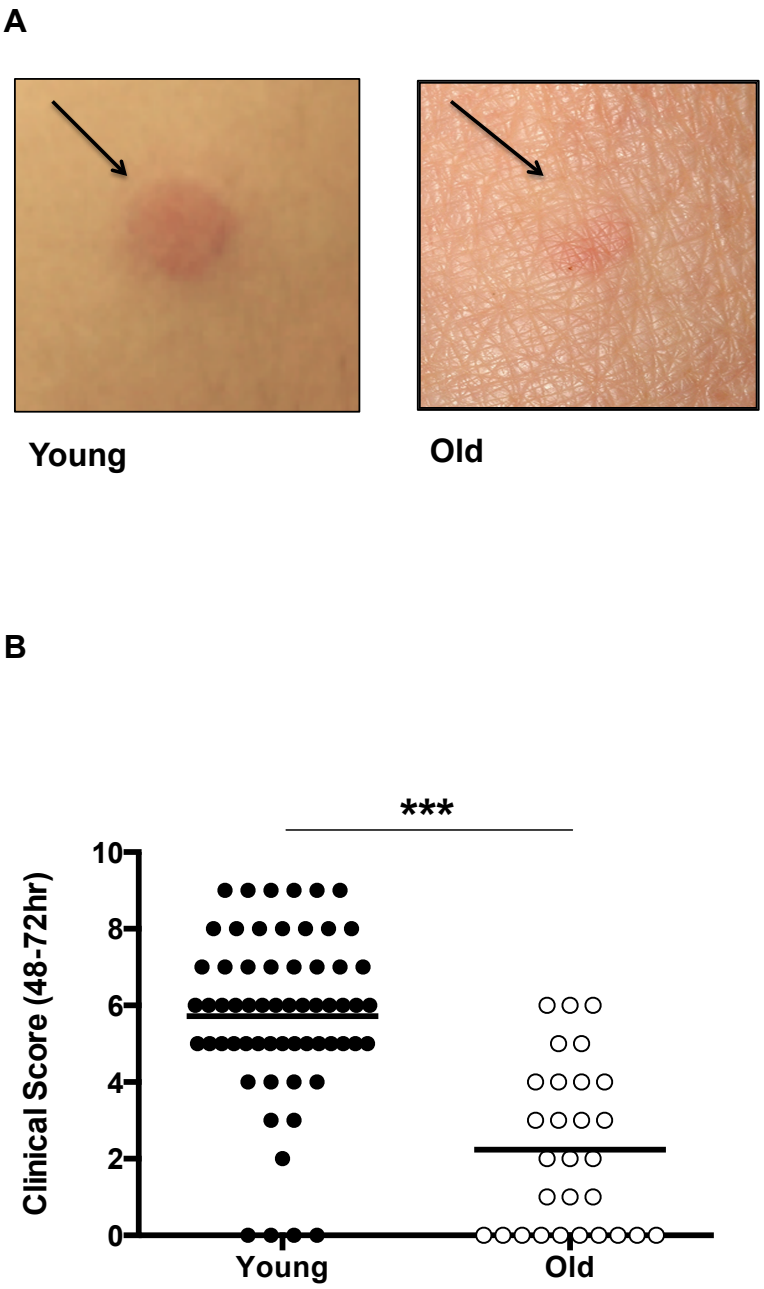


Figure 3.1 Cutaneous clinical responses to VZV antigen in young and old individuals.

Old (n=29) and young (n=61) volunteers were injected with 0.02ml VZV antigen solution intradermally. The clinical response was assessed at 48-72 hours for erythema, diameter of induration and ease of palpability and a clinical score was assigned based on these parameters. (A) A photograph of the clinical response to VZV skin test antigen 3 days after the injection in a young and an old individual. (B) Graph shows cumulative data (n=90) and mean of clinical score in old and young individuals. The clinical score was significantly reduced in the old compared to the young volunteers (***p=<0.0001, Mann Whitney test).

3.3 Peripheral blood response to VZV lysate

We next investigated whether the reduced cutaneous response to VZV antigen in old volunteers reflected a reduced circulating immunity to VZV. We investigated the proliferative capacity of VZV specific cells and their ability to produce inflammatory cytokines in response to VZV lysate *in vitro*.

Freshly isolated PBMCs from old (n=19) and young (n=19) individuals were stimulated with a range of concentrations of VZV lysate (0-10µl/ml) *in vitro*. Proliferation was measured by tritiated thymidine ([3H] thymidine) incorporation 7 days later. PBMCs from old and young volunteers showed similar maximal proliferative responses to stimulation with VZV lysate (p=0.92, Mann Whitney test) (Figure 3.2).

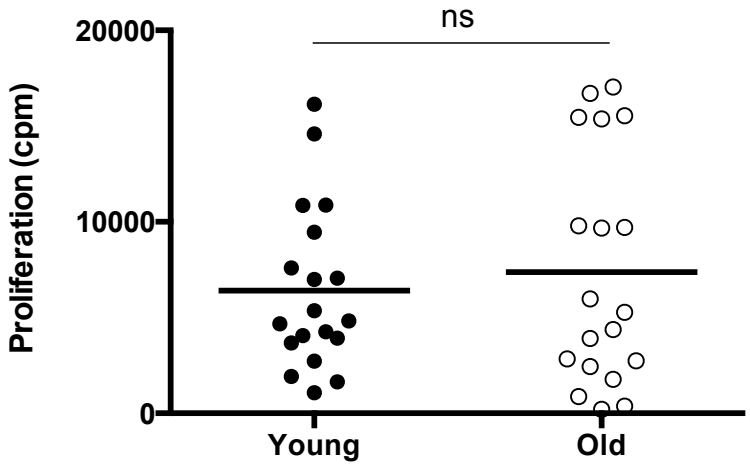


Figure 3.2 PBMC proliferation in response to VZV lysate in vitro assessed by [3H] thymidine incorporation

PBMCs from old (n=19) and young (n=19) individuals were stimulated with a range of VZV lysate concentrations (0-10µl/ml) *in vitro*, with tests performed in triplicate at each concentration. After 7 days, 10µl of 0.0025MBq [3H] thymidine was added and the samples incubated for a further 16 hours. [3H] thymidine incorporation was assessed by liquid scintillation counting. The mean cpm was calculated from the triplicate readings at each lysate concentration used. The maximum mean proliferation value was used for analysis irrespective of the corresponding lysate concentration. Graph shows cumulative data and the mean value for each group. No difference was detected in the maximum level of PBMC proliferation between the old and young volunteers (p=0.92, Mann Whitney test).

However, PBMCs from young individuals showed a greater proliferative response than the old at the lowest VZV lysate concentrations of 0.5 µl/ml (*p=0.04, Mann Whitney test) (Figure 3.3). This suggests that although potential proliferative capacity in the old is equal to that of the young, it does require a higher antigen dose to achieve it. Alternatively, it may reflect a lower number of initial VZV-specific T cells in the circulation.

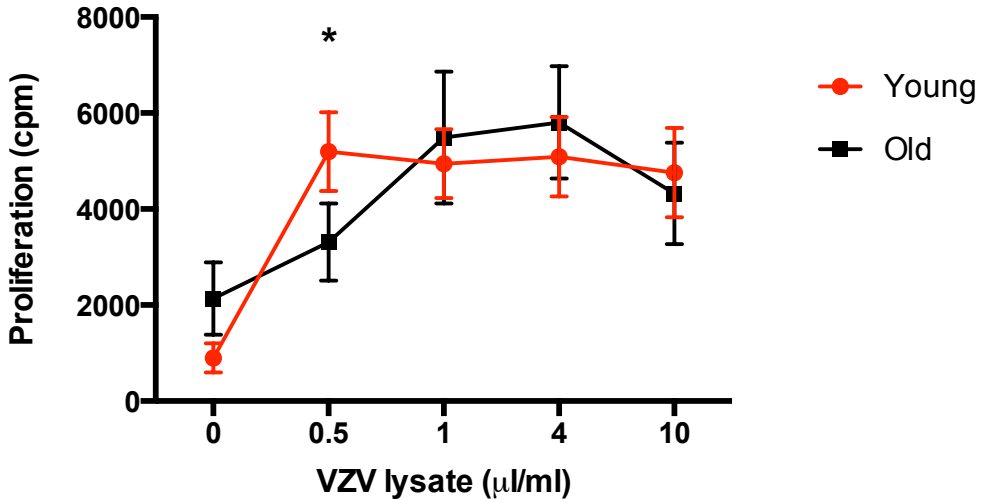


Figure 3.3 PBMC proliferation assessed by [3H] thymidine incorporation in response to *in vitro* stimulation with VZV lysate at a range of concentrations

PBMCs from old (n=19) and young (n=19) individuals were stimulated with a range of VZV lysate concentrations (0-10µl/ml) *in vitro*, with tests performed in triplicate at each concentration. Graph shows cumulative data with the mean value and SEM for each group at a given dose. The young only show greater proliferation at the lowest dose compared to the old volunteers (*p=0.04 Mann Whitney test).

To determine if the frequency of VZV-specific T cells was reduced in the old compared to the young, we assessed Ki67 expression in responding cells at an earlier time point after stimulation. This also allowed us to distinguish between responding $CD4^+$ and $CD8^+$ T cells. Freshly isolated PBMCs from old (n=21) and young (n=18) individuals were stimulated with VZV lysate (4µl/ml) *in vitro* and incubated for 3 days before staining for Ki67 expression.

No difference was found in Ki67 expression in $CD4^+$ or $CD8^+$ T cell subsets between the young and old volunteers (p=0.95 and 0.82 respectively, Mann Whitney test) (Figure 3.4) suggesting that the frequency of VZV-specific T cells is similar in the old and young.

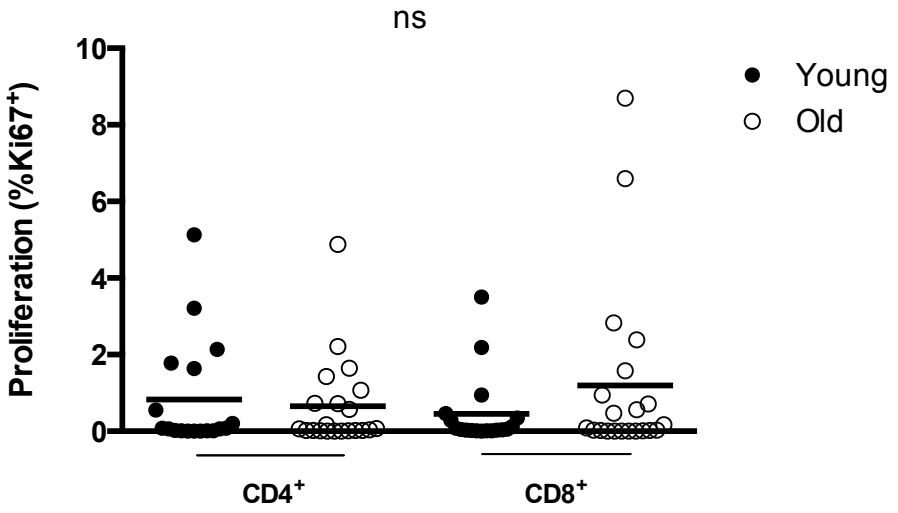


Figure 3.4 PBMC proliferation assessed by Ki67 expression in response to stimulation with VZV lysate *in vitro*

PBMCs from old (n=21) and young (n=18) individuals were stimulated with VZV lysate (4μl/ml) *in vitro*, incubated for 3 days and then stained for Ki67 expression. Samples were analysed by flow cytometry. The graph shows cumulative data and the mean values for each age group and subset. There was no difference in Ki67 expression in either $CD4^+$ or $CD8^+$ T cell subsets between old and young individuals (p=0.95 and 0.82 respectively, Mann Whitney test).

The function of VZV specific $CD4^+$ T cells in peripheral blood was investigated by their ability to produce cytokines in response to *in vitro* stimulation with VZV lysate. Freshly isolated PBMCs from old (n=20) and young (n=20) individuals were stimulated with 40μl/ml VZV lysate for 15 hours in the presence of Brefeldin A. Samples were stained for expression of IFN-γ and IL-2. There were significantly fewer IFN-γ^+CD4^+ T cells identified from the old volunteers compared to the young (p=0.04, Mann Whitney test) (Figure 3.5).

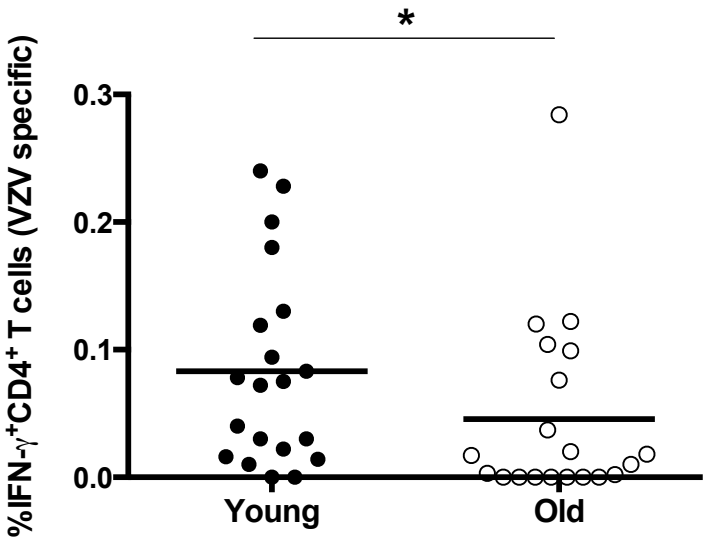


Figure 3.5 Frequency of VZV specific $CD4^+$ T cells by intracellular cytokine staining

PBMCs from old (n=20) and young (n=20) individuals were stimulated with VZV lysate (40μl/ml) *in vitro* for 15 hours in the presence of Brefeldin A. Cells were stained for IFN-γ and IL-2 and analysed by flow cytometry. Graph shows cumulative data and the mean values for each group. The percentage of IFN-γ^+CD4^+ T cells (VZV-specific cells) was significantly lower in the old individuals compared to the young (*p=0.04, Mann Whitney test). IL-2^+CD4^+ T cells were not present in significant numbers compared to non-stimulated controls.

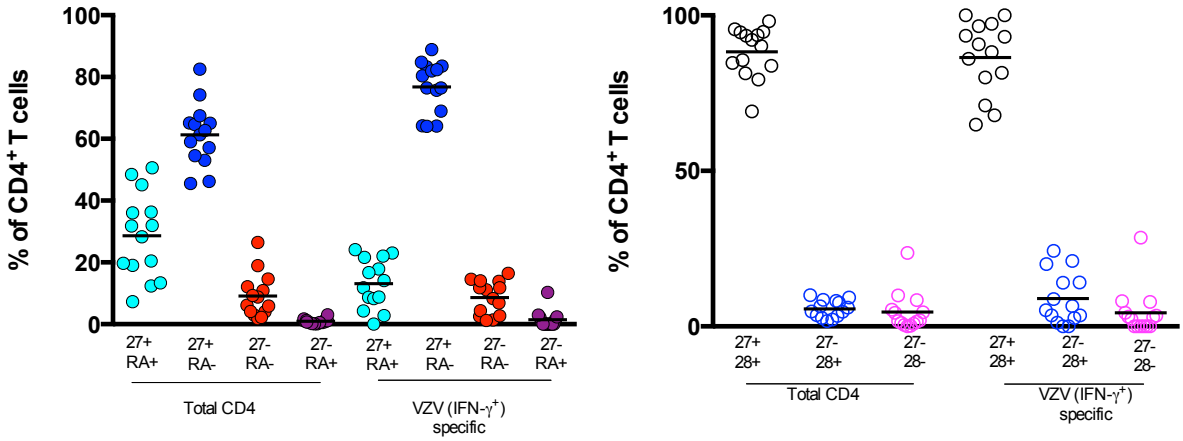
Neither IL-2^+CD4^+ T cells, nor IFN-γ^+ or IL-2^+ $CD8^+$ T cells were found in high enough numbers to quantitate compared to non-stimulated controls in either age group at the time point investigated. IL-2 production, although low at this early stage, is adequate to support subsequent T cell proliferation in both the old and young (Figures 3.2 and 3.4). The above results demonstrate that while the number of VZV specific T cells in peripheral blood appears to be equal in the young and old, the function of these cells assessed by cytokine secretion is impaired in the old volunteers, highlighted by the reduced proportion of IFN-γ^+CD4^+ T cells after stimulation *in vitro* in the old, confirming previous reports (Asanuma, Sharp et al. 2000; Patterson-Bartlett, Levin et al. 2007).

3.4 Differentiation status of VZV-specific T cells in peripheral blood

To further characterise the VZV-specific $CD4^+$ T cells in the peripheral blood we examined their differentiation status. PBMCs were isolated from blood sample from old and young volunteers. VZV-specific $CD4^+$ T cells were identified by their ability to synthesise IFN-γ after overnight stimulation with VZV lysate as described above. Cells were also stained for the presence of CD27, CD28, and CD45RA allowing examination of differentiation state. The data on the elderly cohort was gathered previously by another member of our group, Dr Sarah Jackson (Figure 3.6). The majority of VZV-specific T cells were of central memory phenotype ($CD45RA^-CD27^+$) and were not a highly differentiated population ($CD27^+CD28^+$) in both the young and old groups (Figure 3.6).

A

OLD



B

YOUNG

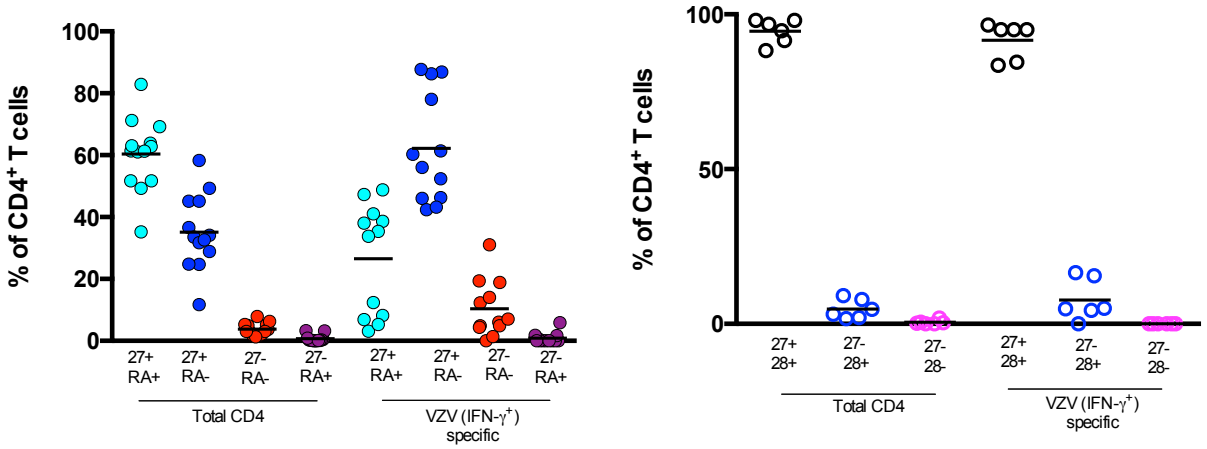


Figure 3.6 Differentiation state of peripheral blood VZV specific $CD4^+$ T cells

PBMCs were isolated from peripheral blood samples taken from young (n=12) and old (n=14) volunteers and stimulated for 15 hours with VZV lysate in the presence of Brefeldin A. Cells were stained for expression of IFN-γ to identify VZV specific T cells as well as CD27, CD28 and CD45RA, key differentiation markers. Samples were analysed by flow cytometry. (A, B) Graphs show cumulative data. Bars show mean values. Each symbol represents one individual. VZV-specific T cells show a predominantly central memory like phenotype ($CD45RA^-CD27^+$) in both old and young volunteers. The majority of VZV-specific T cells in peripheral blood are not highly differentiated ($CD27^+CD28^+$).

3.5 Discussion

This work has demonstrated that the skin DTH response to VZV skin test antigen is reduced in old individuals compared to young and this is in keeping with previous work (Sadaoka, Okamoto et al. 2008). We also confirm that the function of VZV–specific cells is impaired in the old individuals demonstrated by significantly fewer $CD4^+IFN\text{-}\gamma^+$ T cells after *in vitro* stimulation with VZV lysate compared to in the young (Asanuma, Sharp et al. 2000; Patterson-Bartlett, Levin et al. 2007; Levin, Oxman et al. 2008; Sadaoka, Okamoto et al. 2008). However, in contrast to studies that have used responder cell frequency to report a reduced number of circulating VZV specific T lymphocytes in the elderly (Levin, Oxman et al. 2008) we found that the number of cells entering cell cycle soon after stimulation assessed by Ki67 expression was similar between the two groups. This suggests that the number of circulating VZV-specific cells is not different between the groups. Other work has shown an increase in the number of VZV-specific cells in the elderly (Patterson-Bartlett, Levin et al. 2007).

$CD4^+IL\text{-}2^+$ VZV-specific cells were not seen in high enough numbers to quantitate in either age group. IL-2 production is predominantly seen during activation of naïve T cells and T_{CM} cells more so than T_{EM} cells (Sallusto, Lenig et al. 1999). IL-2 was present in the supernatant of VZV-stimulated blood lymphocytes from immune adults (Hayward, Cosyns et al. 1998). Other studies have shown that lymphocytes from elderly individuals produce significantly less IL-2 after stimulation (Gillis, Kozak et al. 1981; Huang, Pechere et al. 1992; Rink, Cakman et al. 1998). It is possible that increasing our sample size would allow us to detect such a difference between the two groups if it exists.

VZV-specific $CD8^+$ T cells did not produce high enough levels of either IL-2 or IFN-γ to be identified in this work. The scarcity of cytokine secreting VZV-specific $CD8^+$ T cells has been reported in previous studies (Asanuma, Sharp et al. 2000; Patterson-Bartlett, Levin et al. 2007). This remains the case even when a number of different VZV antigens have been used to stimulate VZV-

specific T-cells with the majority of responses being mediated by $CD4^+$ T cells (Jones, Black et al. 2006). This relative lack of VZV-specific $CD8^+$ T cells may reflect the known MHC class I down-regulation induced by VZV (Abendroth, Lin et al. 2001). However, $CD8^+IFN\text{-}\gamma^+$ cells are shown to increase in old individuals after receiving the shingles vaccine but returned close to baseline by 6 weeks after vaccination, suggesting this is a tightly regulated population (Patterson-Bartlett, Levin et al. 2007). The proliferation of $CD8^+$ T cell seen by Ki67 expression may reflect in part a bystander effect (Tough, Borrow et al. 1996; Tough, Zhang et al. 2001; Koschella, Voehringer et al. 2004).

We investigated the differentiation status of total and VZV-specific T cells by examining the expression of CD45RA, CD27 and CD28. Previous work by our group has shown that subsets identified by CCR7 and CD27 were very similar although not completely overlapping (Libri, Azevedo et al. 2011). We used CD27 in combination with CD45RA to discriminate between the T cell subsets: naïve ($CD45RA^+CD27^+$), central memory ($CD45RA^-CD27^+$), effector memory ($CD45RA^-CD27^-$) and CD45RA re-expressing effector memory cells ($CD45RA^+CD27^-$). As would be expected, the old volunteers had a smaller naïve ($CD45RA^+CD27^+$) and larger memory population in the total T cell pool compared to the young. The majority of VZV-specific cells in both old and young volunteers are of predominantly central memory phenotype ($CD45RA^-CD27^+$) and are not highly differentiated ($CD27^+CD28^+$). Large central memory populations of VZV-specific T cells that are not highly differentiated have also been identified in other studies (Patterson-Bartlett, Levin et al. 2007; Malavige, Jones et al. 2008). There were also significant naïve-like ($CD45RA^+CD27^+$) populations in the VZV-specific T cell populations in both young and old volunteers in keeping with the possibility that some central memory cells can re-express CD45RA (Geginat, Lanzavecchia et al. 2003; Dunne, Belaramani et al. 2005). The purpose of re-expression of CD45RA in such a scenario is unclear. Given that VZV is proposed to undergo clinically silent reactivation relatively frequently (Quinlivan, Ayres et al. 2007; Malavige, Jones et al. 2008) it might be expected, contrary to our observations, that in

the old a greater proportion of the VZV-specific cells would be more highly differentiated.

There are several possible reasons why VZV-specific T cells in the elderly may display reduced IFN-γ production e.g. effector $CD4^+$ T cell functions may come under increased suppression as a result of the increased proportion of circulating Tregs in the elderly (Trzonkowski, Szmit et al. 2006; Vukmanovic-Stejic, Zhang et al. 2006); raised type 1 interferon signalling in the process of inflamm-ageing (Giunta 2008) may suppress virus specific $CD4^+$ T cell function (Wilson, Yamada et al. 2013); VZV-specific cells may be out-competed by other memory expansions present in the old (Khan, Hislop et al. 2004).

The reduced clinical DTH response to VZV antigen in the skin of the old volunteers could be attributed to a reduced number of functioning VZV specific T cells being available for recruitment from the circulation into the skin. In the next chapter we will explore this further by comparing the cellular infiltrate at the site of the DTH response between young and old volunteers.

Chapter 4: Cellular cutaneous response to VZV antigen in old and young individuals

4.1 Introduction

In the last chapter we showed that old volunteers show a reduced clinical response to VZV antigen in the skin compared to young volunteers. We also showed that this did not reflect the number of circulating VZV-specific T cells which were similar in the two age groups. However, there are many other cells involved in the induction of a recall response in the skin, given that it is an integrated multistep process. Therefore in this chapter we compared the kinetics of the cellular response to VZV antigen in the skin of old and young individuals. We did this by taking skin biopsies at various time points after VZV antigen injection into the skin allowing us to study the *in vivo* events as they unfolded.

4.2 Cutaneous T cell response to VZV antigen

4.2.1 T cell accumulation at site of cutaneous VZV challenge

Since DTH responses are dependent on antigen-specific $CD4^+$ T cells we first investigated whether the reduced clinical response to the VZV antigen in the old individuals was associated with a reduced infiltration of lymphocytes. We took 5mm punch biopsies of normal skin and at 1, 3 and 7 days after VZV antigen injection at the site of the intradermal injection, from at least 3 young and 3 old volunteers for each time point. Haematoxylin and eosin staining (kindly performed by technician staff, Histopathology Department, Royal Free Hospital) of skin sections taken after VZV antigen injection from old and young volunteers shows the presence of large, dense predominantly perivascular lymphocytic infiltrates in the young individuals, and these are considerably larger than those seen in the old at all time points (Figure 4.1).

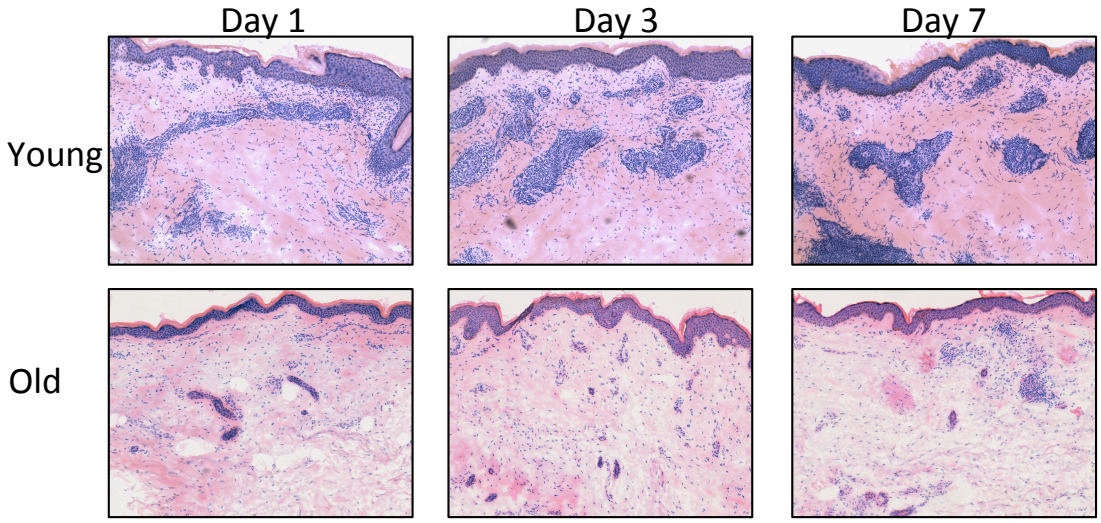


Figure 4.1 Lymphocytic infiltrate at the site of the cutaneous DTH response in old and young volunteers

Skin sections from biopsies taken 1, 3 and 7 days after VZV antigen injection from old and young volunteers were stained with haematoxylin and eosin (x10). Large dense predominantly perivascular lymphocytic infiltrates are seen in the young at days 1, 3 and 7 post VZV skin test. Although present in the old, the perivascular infiltrates are considerably smaller in size.

To characterise the infiltrate further we performed immunofluorescence staining to identify $CD4^+$ and $CD8^+$ T cell subsets. For each section, the five largest perivascular infiltrates were photographed and the number of cells counted in each frame. The mean value of the cell count for the 5 frames was calculated and used for analysis. There is an increase in the numbers of $CD4^+$ T cells and $CD8^+$ T from day 0 to day 7 in the young ($p=0.0005$ and $p=0.0007$ respectively, Kruskal Wallis test) but not the old (Figures 4.2 and 4.3), with the trend visible in the young by day 1 post injection.

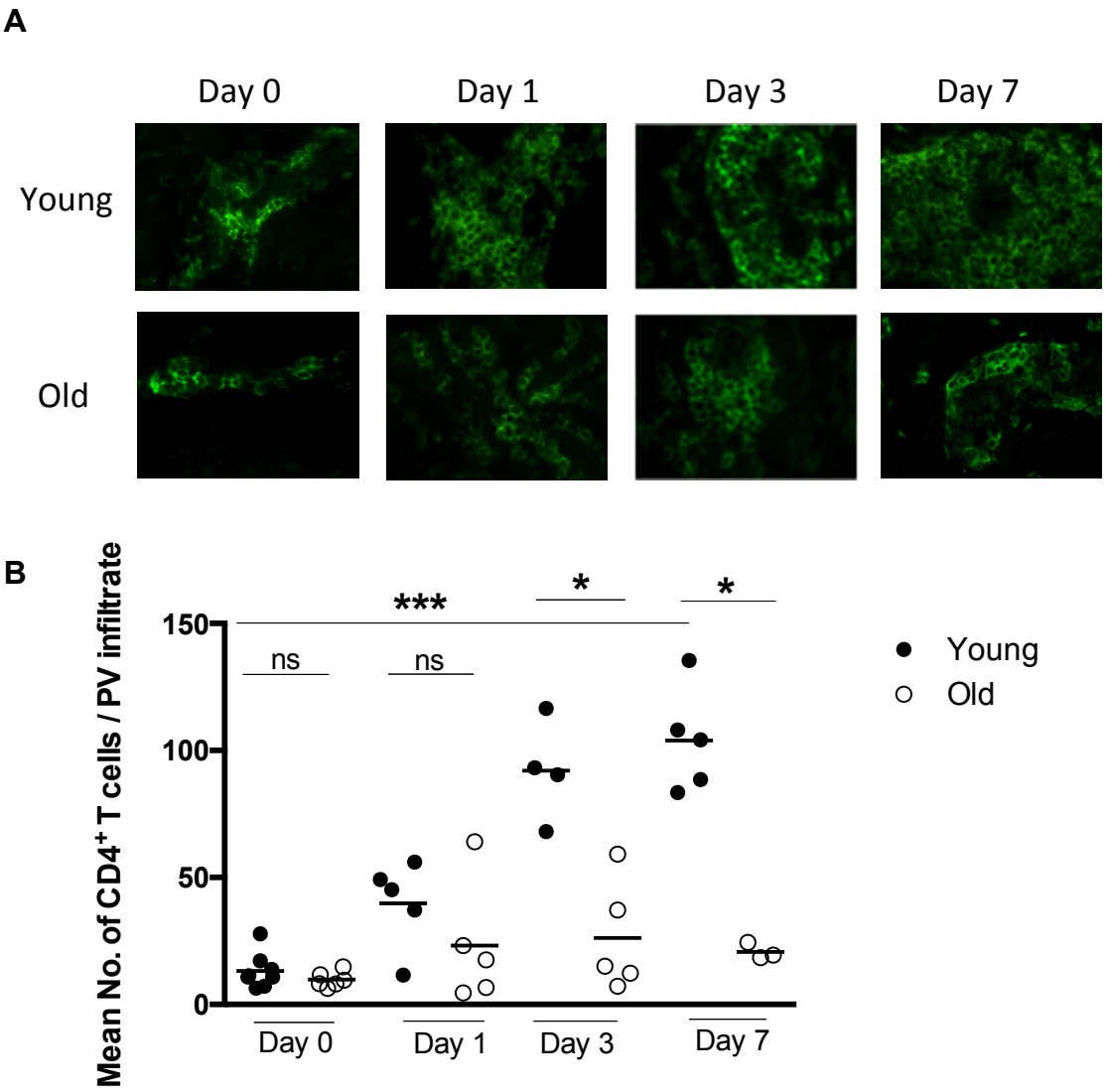


Figure 4.2 $CD4^+$ T cell accumulation at the site of the cutaneous DTH response in young and old volunteers

Immunofluorescence staining to identify $CD4^+$ T cells was performed on normal skin sections and from biopsies taken 1, 3 and 7 days after cutaneous challenge with VZV antigen from old and young volunteers (n=3-7 per age group at each time point). The five largest perivascular infiltrates per section were photographed and counted. The mean of these counts was used for analysis for each individual. (A) Representative images are shown of $CD4^+$ T cell infiltrates in old and young volunteers before and after VZV injection. (B) Graph of cumulative data and mean is shown. Each symbol represents one individual. $CD4^+$ T cells numbers increase significantly over the time course in the young (***p=0.0005, Kruskal Wallis test) but not in the old individuals. There is a significant difference in the number of $CD4^+$ T cells in the young compared to the old individuals by days 3 and 7 after VZV antigen injection (*p=0.02 and *p=0.04 respectively, Mann Whitney test).

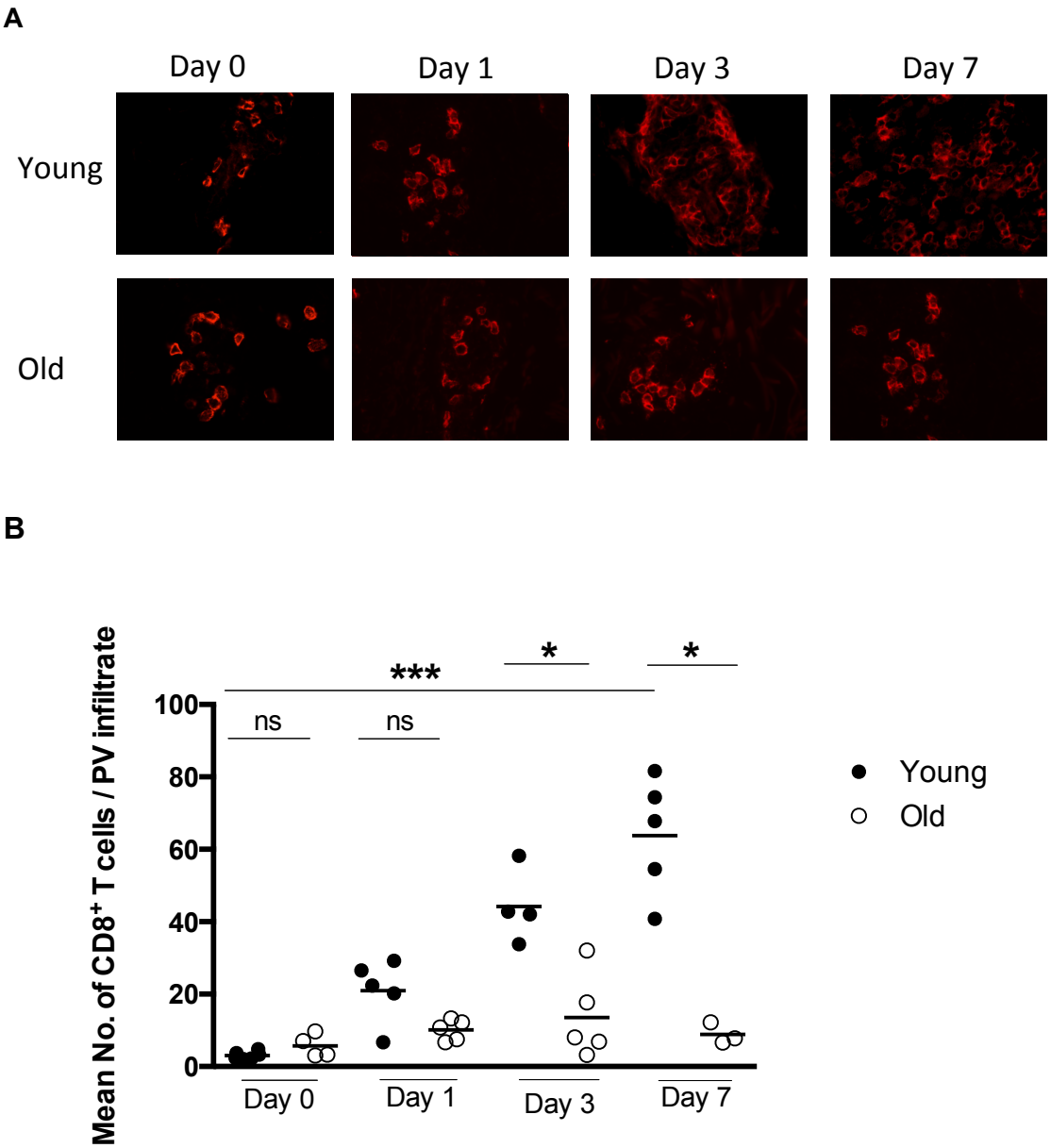


Figure 4.3 $CD8^+$ T cell accumulation at the site of the cutaneous DTH response in young and old volunteers

Immunofluorescence staining to identify $CD8^+$ T cells was performed on normal skin sections and from biopsies taken 1, 3 and 7 days after intradermal challenge with VZV antigen from old and young volunteers (n=3-5 per age group at each time point). The five largest perivascular infiltrates per section were photographed and counted. The mean of these counts was used for analysis for each individual. (A) Representative images are shown of $CD8^+$ T cells infiltrates in old and young volunteers before and after VZV injection. (B) Graph of cumulative data and mean is shown. Each symbol represents one individual. The number of $CD8^+$ T cells increases significantly over the time course in the young (***p=0.0007, Kruskal Wallis test) but not in the old volunteers. There is a significant difference in the number of $CD8^+$ T cells in the young compared to the old by days 3 and 7 after VZV antigen injection (*p=0.02 and *p=0.04 respectively, Mann Whitney test).

Interestingly the number of $CD4^+$ T cells infiltrating the skin in response to VZV antigen is greatest at day 7 in the young cohort even though the peak clinical response is seen at 48-72 hours. However, the clinical score does correlate with the number of infiltrating $CD4^+$ T cells seen 3 and 7 days after the VZV injection ($p<0.0001$, linear regression analysis) (Figure 4.4).

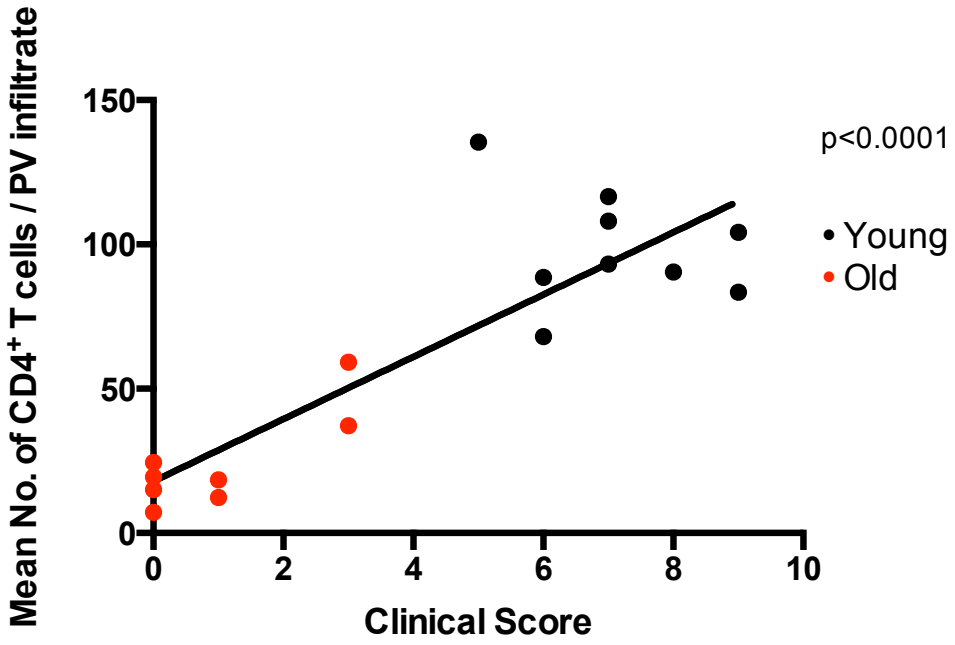


Figure 4.4 Correlation between clinical score and mean number of $CD4^+$ T cells / PV infiltrate

Immunofluorescence staining was performed on skin sections taken from old and young volunteers at days 3 and 7 after cutaneous challenge with VZV antigen to identify $CD4^+$ T cells. The five largest perivascular infiltrates per section were photographed and counted. The mean of these counts was used for analysis for each individual. Graph shows positive correlation between the mean number of $CD4^+$T cells / PV infiltrate and clinical score ($p<0.0001$, linear regression analysis).

4.2.2 T cell proliferation at site of cutaneous VZV challenge

Our group has previously shown that T cell proliferation is likely to occur in the skin during the Mantoux response in humans (Vukmanovic-Stejic, Reed et al. 2006) contributing to T cell accumulation at the site of injection. Therefore one factor that may contribute to the lack of T cell accumulation after VZV injection in the skin of the old is reduced proliferative capacity of VZV-specific T cells compared to the young. We performed immunofluorescence staining to identify expression of Ki67 in $CD4^+$ and $CD8^+$ T cells on skin sections taken 1,3 and 7 days after VZV antigen injection from old and young volunteers. There was significant local proliferation of $CD4^+$ and $CD8^+$ T cells in the young in the skin over the time course (***p=0.0002 and **p=0.002 respectively, Kruskal Wallis test) but not in the old individuals (Figure 4.5 and 4.6 respectively). Maximum proliferation occurred in the young at day 7 with an average of 20% of $CD4^+$ T cells and 5-10% of $CD8^+$ T cells being in cell cycle. Despite similar numbers of $CD4^+$ and $CD8^+$ T cells being present in the skin 24 hours after the VZV skin test in both age groups, in the young there is subsequently significant local proliferation of these cells that is not seen in the elderly. This indicates that either there is a true reduction in proliferative capacity of antigen-specific T cells in the skin of the elderly during the DTH response, or that there are inadequate numbers of antigen specific cells present early in the response available to undergo proliferation, possibly due to impaired recruitment from the circulation.

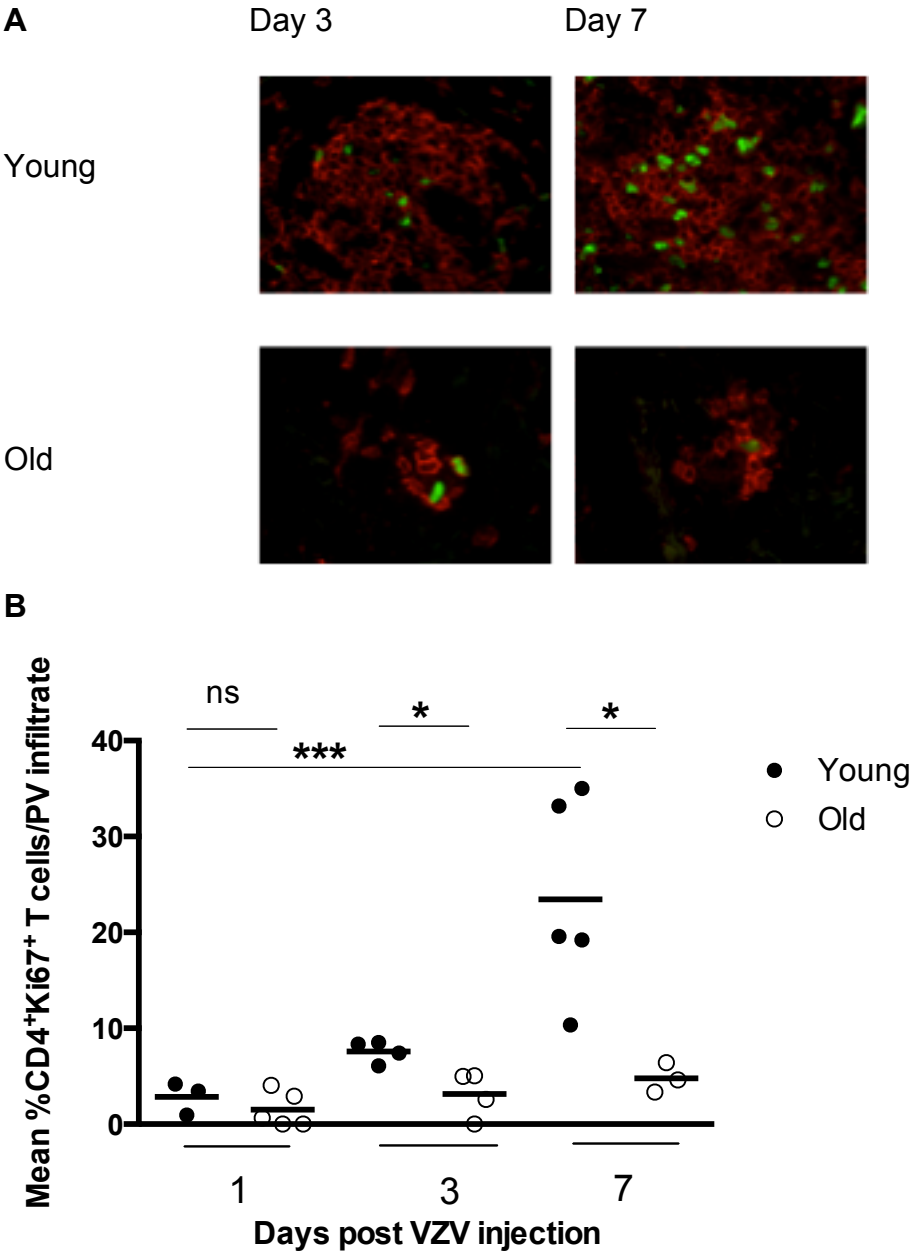


Figure 4.5 Proliferation of $CD4^+$ T cells at the site of the cutaneous DTH response

Immunofluorescence staining for CD4 and Ki67 expression was performed on skin sections from biopsies taken 1, 3, and 7 days after cutaneous challenge with VZV antigen from old and young volunteers (n=3-5 per age group at each time point). The % of double positive cells in the five largest perivascular infiltrates was calculated and the mean of these 5 readings was used for analysis for each individual. (A) Representative images are shown for day 3 and day 7 skin sections in old and young volunteers with cells stained for CD4 (red) and Ki67 (green). (B) Graph shows cumulative data and mean for each group. Each symbol indicates one individual. There is a significant increase in the mean % of $CD4^+Ki67^+$ T cells over the time course in the young (***0.0002, Kruskal Wallis test) but not in the old individuals. There are significantly more proliferating $CD4^+$ T cells in the young cohort compared to the elderly, 3 and 7 days after VZV antigen injection (*p=0.02 and *p=0.03 respectively, Mann Whitney test).

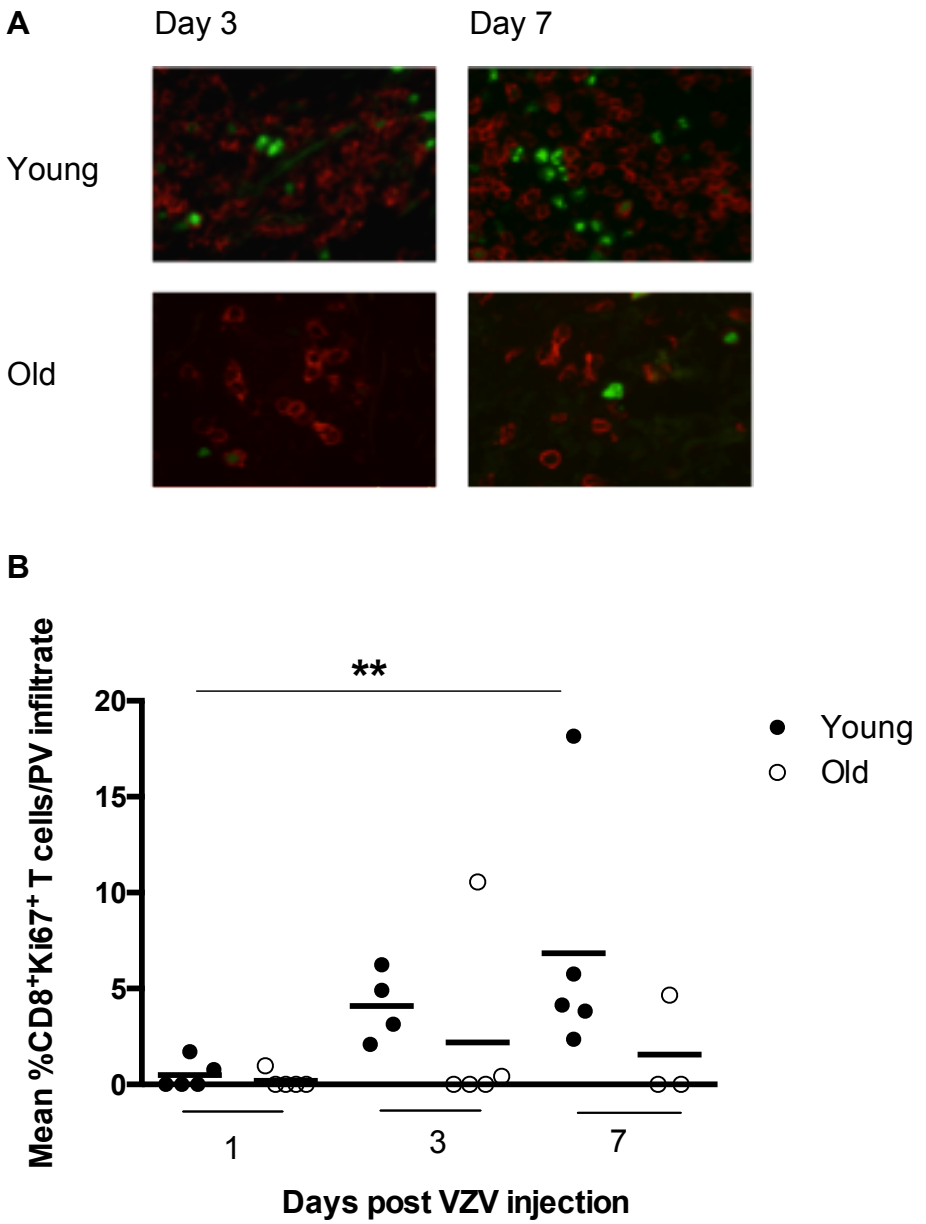


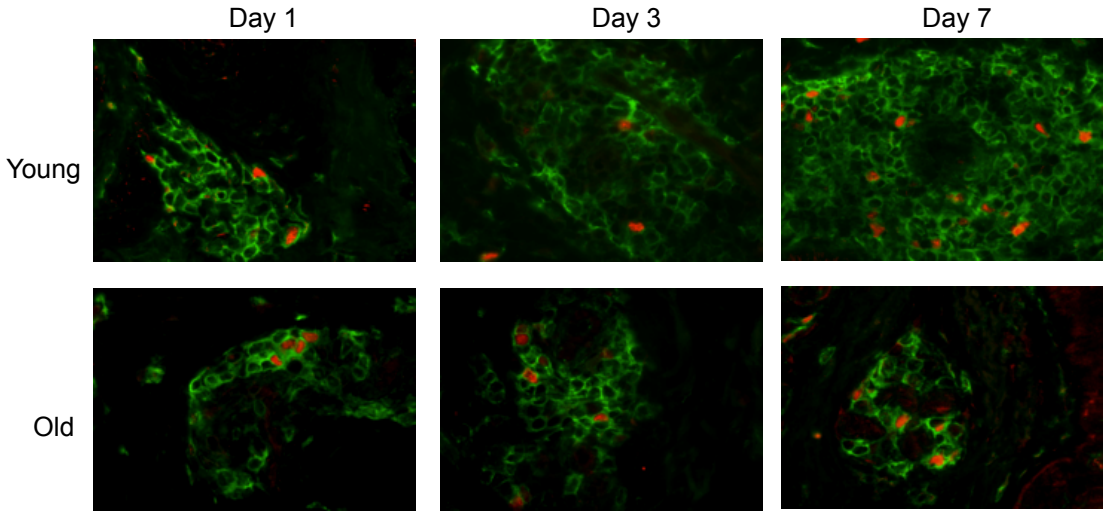
Figure 4.6 Proliferation of $CD8^+$ T cells at the site of the cutaneous DTH response

Immunofluorescence staining for CD8 and Ki67 expression was performed on skin sections from biopsies taken 1, 3, and 7 days after cutaneous challenge with VZV antigen from old and young volunteers (n=3-5 per age group at each time point). The % of double positive cells in the five largest perivascular infiltrates was calculated and the mean of these 5 readings was used for analysis for each individual. (A) Representative images are shown for skin sections taken 3 and 7 days after VZV antigen injection from old and young volunteers with cells stained for CD8 (red) and Ki67 (green). (B) Graph shows cumulative data and the mean for each group. Each symbol represents one individual. There is a significant increase in the mean % of proliferating $CD8^+$ T cells in young individuals over the time course (**p=0.002, Kruskal Wallis test) not present in the elderly.

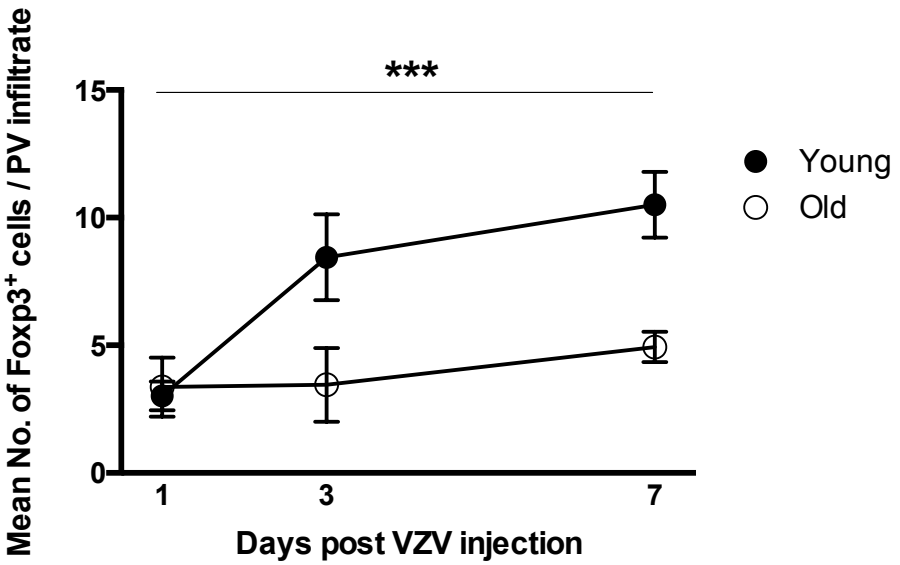
4.2.3 Accumulation of $CD4^+Foxp3^+$ T cells at site of cutaneous VZV challenge

Older individuals have an increased proportion of Tregs in normal skin (Agius, Lacy et al. 2009). Tregs are known to have a suppressive effect on immune responses and we hypothesised that they may play a role in dampening the cellular response to VZV antigen in the skin of the old volunteers e.g., by reducing proliferation of antigen-specific T cells. We therefore compared the accumulation of Tregs in the skin of old and young individuals during the DTH response to VZV antigen. We performed immunofluorescence staining to identify $CD4^+$ and $Foxp3^+$ T cells on skin sections from biopsies taken 1, 3 and 7 days after VZV antigen injection from old and young volunteers. There was an increase in the mean number of $CD4^+Foxp3^+$ T cells per perivascular infiltrate over the time course in the young but not the old volunteers ($p=0.0005$, Kruskal Wallis test, Figure 4.7) reflecting the trend for the mean number of $CD4^+$ T cells. Therefore the proportion of $CD4^+Foxp3^+$ T cells remained relatively stable in both old and young volunteers over the time course and the proportion of $CD4^+Foxp3^+$ T cells remained significantly higher in the old volunteers compared to the young throughout (D1 $^{**}p=0.008$, D3 $^{*}p=0.02$, D7 $^{*}p=0.02$ respectively, Mann Whitney test).

A


Day 1
Day 3
Day 7
Young
Old

B


***
15
10
5
0
1
3
7
Mean No. of Foxp3+ cells / PV infiltrate
Days post VZV injection
Young
Old

C

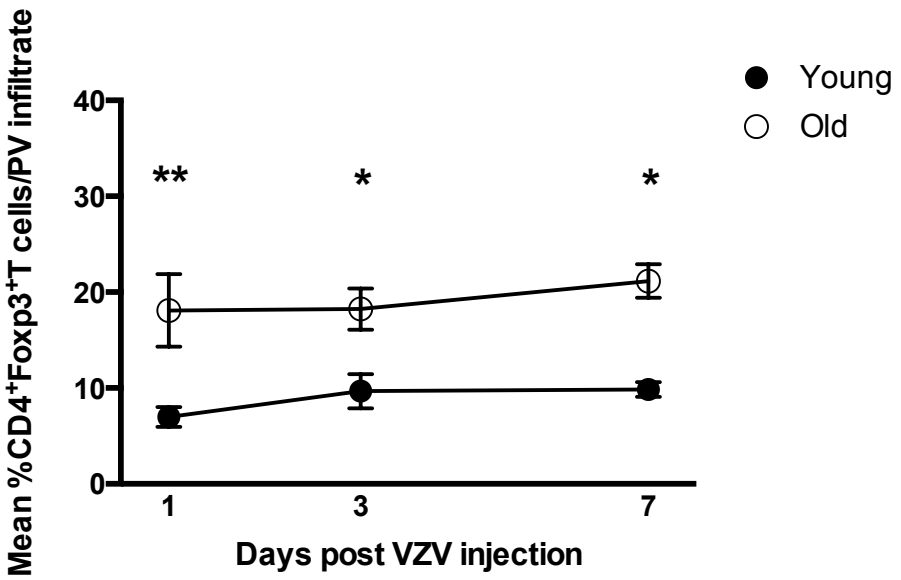


Figure 4.7 Accumulation of $CD4^+Foxp3^+$ T cells at the site of the cutaneous DTH response in young and old volunteers

Immunofluorescence staining for CD4 and Foxp3 expression was performed on skin sections from biopsies taken 1, 3 and 7 days after VZV antigen injection from old and young volunteers. The % of double positive cells in the five largest perivascular infiltrates was calculated and the mean of these 5 readings was used for analysis for each individual. (A) Representative images are shown of skin sections at each time point from old and young volunteers stained for CD4 (green) and Foxp3 (red). (B) Graph shows mean of data for each age group at stated time points (n=3-5 per age group at each time point) +/- SEM. There is a significant increase in the mean number of $CD4^+Foxp3^+$ T cells in perivascular infiltrates in young individuals over the time course (***p=0.0005, Kruskal Wallis test). Significant accumulation of $CD4^+Foxp3^+$ T cells is not seen in the old cohort. (C) Graph shows mean of data for each age group at stated time points (n=3-5 per age group at each time point) +/- SEM. The mean proportion of $CD4^+Foxp3^+$ T cells remains relatively stable over the time course in young and old volunteers. The mean proportion of $CD4^+Foxp3^+$ T cells remains significantly higher in the old compared to the young at all stated time points (day 1 **p=0.008, day 3 *p=0.02, day 7 *p=0.02 respectively, Mann Whitney test).

To determine if the accumulation of $CD4^+Foxp3^+$ T cells at least in the young was due in part to local proliferation mirroring the accumulation of the total $CD4^+$ T cell population, we performed immunofluorescence staining for Foxp3 and Ki67 expression on skin sections from biopsies taken 1, 3 and 7 days after VZV skin test from young volunteers. Proliferating $Foxp3^+$ T cells were

identified by overlap of these two stains (Figure 4.8) providing evidence of some local proliferation of $Foxp3^+$ T cells over the time course.

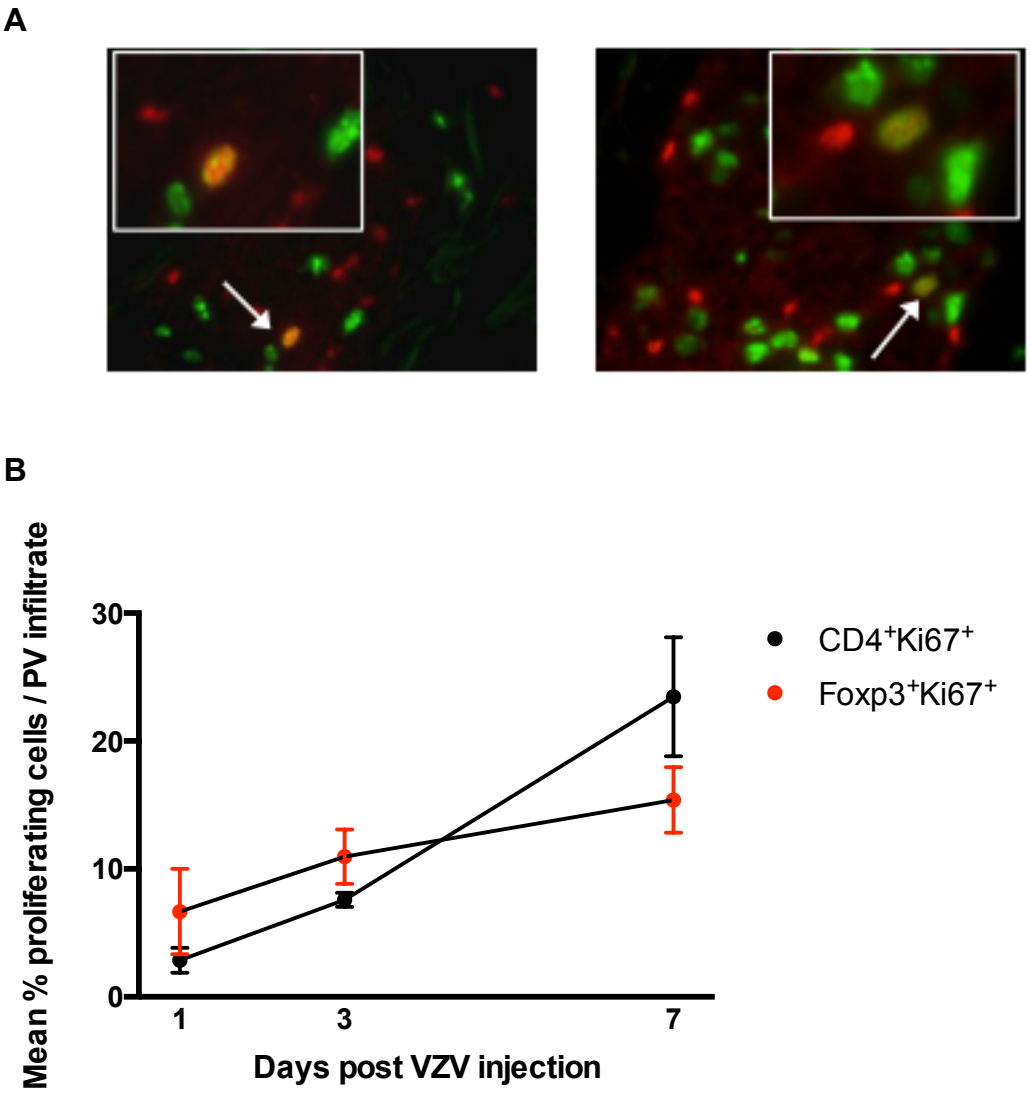


Fig 4.8 Proliferation of $CD4^+Foxp3^+$ T cells at the site of the cutaneous DTH response

Immunofluorescence staining for Foxp3 and Ki67 expression was performed on skin sections from biopsies taken 1, 3, and 7 days after cutaneous challenge with VZV antigen in young volunteers, to identify proliferating $CD4^+Foxp3^+$ T cells. The % of double positive cells in the five largest perivascular infiltrates was calculated and the mean of these 5 readings was used for analysis for each individual (A) Representative images of co-staining are shown Foxp3 (red) and Ki67 (green). (B) Graph shows cumulative data, mean and SEM for each group (n=3-5 per age group at each time point). There is a trend for the mean % of proliferating $CD4^+Foxp3^+$ to increase over the time course as seen in the total $CD4^+$ T cell population.

We compared normal skin from old individuals who typically mount a poor clinical response and young individuals who mount a robust clinical response and found that this disparity in the proportion of Tregs is present prior to the injection of the VZV antigen (**p=0.006, Mann Whitney test, Figure 4.9) (Agius, Lacy et al. 2009). Interestingly when we examined the normal skin of a small subset of old volunteers who responded to the VZV antigen in a manner similar to young volunteers, the proportion of $CD4^+Foxp3^+$ T cells was significantly lower than in the typical old cohort (*p=0.02, Mann Whitney test) and more in keeping with the young cohort. This implies indirectly that the resident $CD4^+Foxp3^+$ T cells may exert a negative regulatory effect on the subsequent evolution of the cutaneous DTH response.

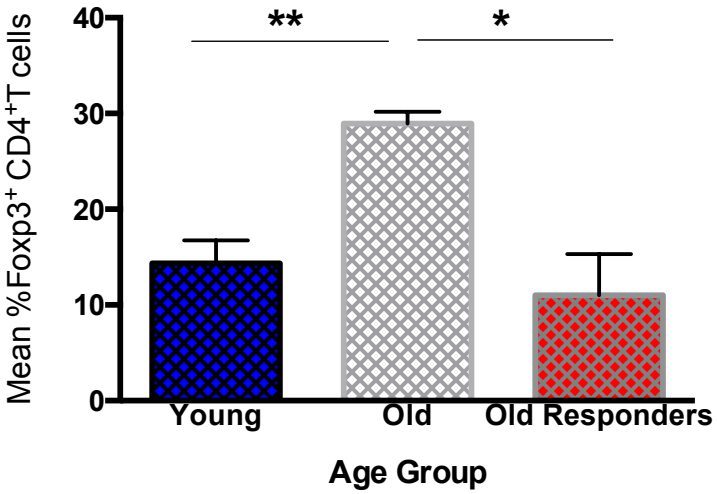


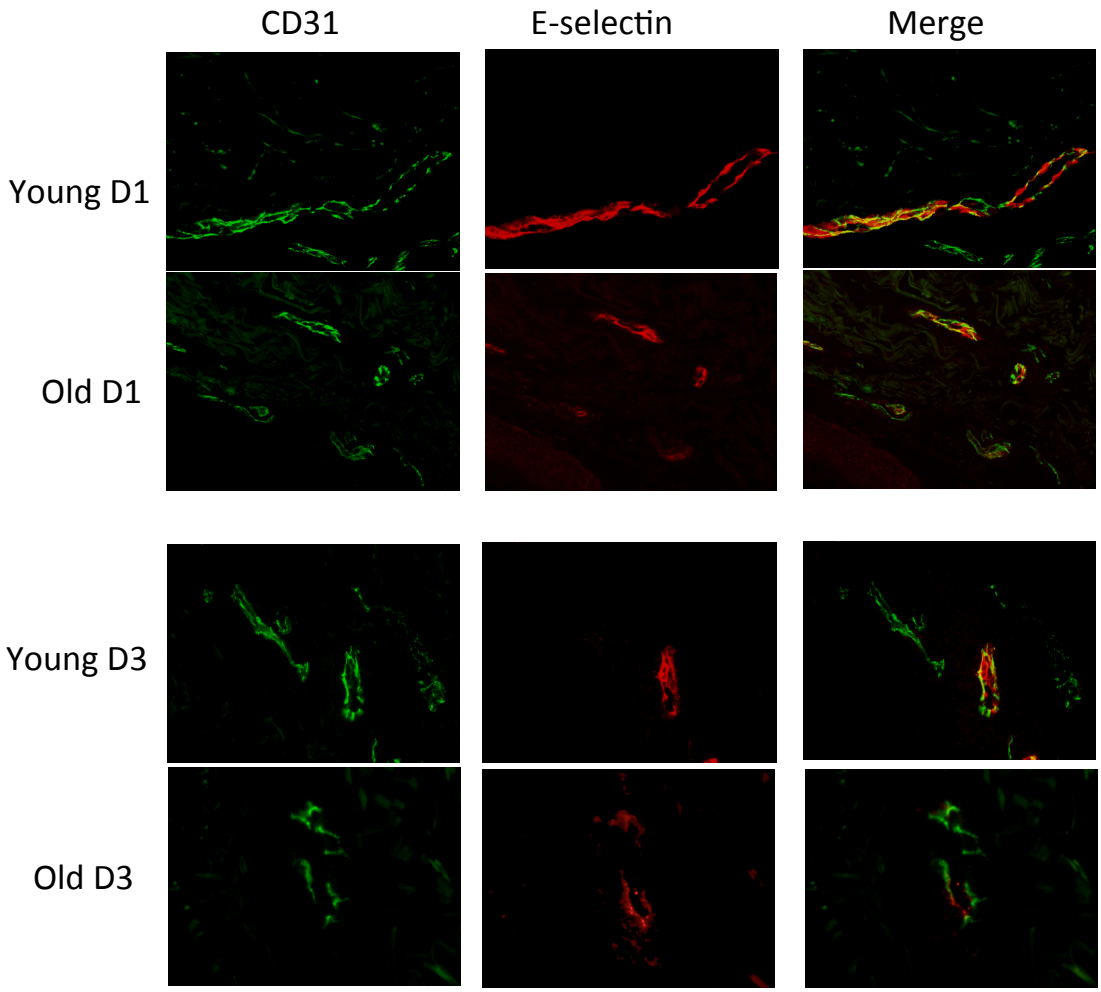
Fig 4.9 Proportion of $CD4^+Foxp3^+$ T cells in normal skin of old and young volunteers

Immunofluorescence staining for CD4 and Foxp3 expression was performed on sections of normal skin taken from old and young volunteers. The % of double positive cells in 5 fields was calculated and the mean of these 5 readings was used for analysis for each individual (n=5-8 for each group). Graph shows the mean +/- SEM of the data for each group. The average proportion of $CD4^+Foxp3^+$ T cells is significantly higher in normal skin of old people compared to young (**p=0.006 Mann Whitney test). The average proportion of $CD4^+Foxp3^+$ T cells in the old group was also significantly higher (*p=0.02, Mann Whitney test) than that seen in a small subset of old volunteers who mounted a strong clinical response to VZV antigen, who had similar proportions of $CD4^+Foxp3^+$ T cells to the young.

4.3 Activation of dermal endothelium at site of the DTH response

We have shown above that local T cell proliferation is likely to contribute in part to antigen-specific T cell accumulation at the site of the DTH response in young individuals. T cell accumulation was not seen in the elderly and may reflect an inability to recruit antigen-specific T cells from the circulation into the skin or inadequate activation of skin resident T cells with failure to induce appropriate proliferation. Pro-inflammatory cytokines such as TNF-α and IL-1 released during innate immune responses to infection or trauma are responsible for activating local endothelium. In the skin, activated dermal endothelium up-regulates expression of cell-adhesion molecules E-selectin ICAM-1 and VCAM-1 promoting binding of circulating antigen-experienced T cells and other leukocytes to the site of inflammation (Groves, Allen et al. 1995). Skin homing memory T cells express corresponding ligands e.g., CLA, which binds E-selectin. Upregulation of E-selectin expression on activated endothelium is seen as early as 2 hours after stimulation with cytokines (Wyble, Hynes et al. 1997), and precedes that of ICAM-1 and VCAM-1 (Bevilacqua 1993). To determine if there was a defect in activation of dermal endothelium in the old volunteers compared to the young after VZV antigen injection we performed immunofluorescence staining for CD31, a cell surface adhesion molecule constitutively expressed on dermal endothelium and E-selectin. We assessed skin sections from biopsies taken 1 and 3 days after VZV antigen injection from old and young volunteers. On average, 20% of dermal blood vessels were activated in the old compared to approximately 75% in the young at both 1 and 3 days after VZV antigen challenge (*p=0.016 and 0.027 respectively, Mann Whitney test) (Figure 4.10). Endothelial activation in the dermis, assessed by expression of E-selectin, is significantly reduced in the old compared to the young and this could impair the ability of T cells to migrate to the site of the DTH response.

A


CD31
E-selectin
Merge
Young D1
Old D1
Young D3
Old D3

B

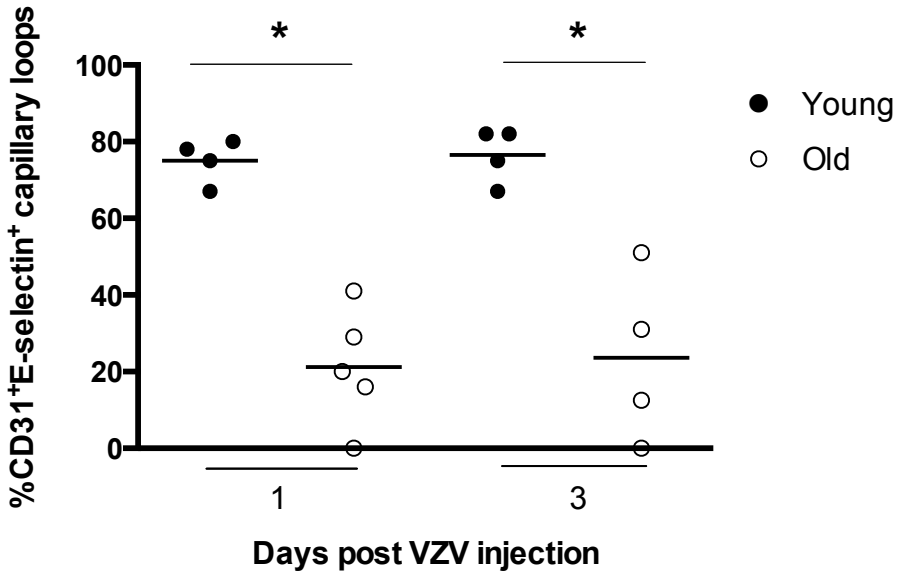

%CD31+E-selectin+ capillary loops
100
80
60
40
20
0
*
*
1
3
Days post VZV injection
Young
Old

Figure 4.10 Activation of dermal endothelium at site of DTH response

Immunofluorescence staining for CD31 and E-selectin expression was performed on skin sections from biopsies taken 1 and 3 days after cutaneous challenge with VZV antigen from young and old volunteers (n=4-5 per age group at each time point). The number of double positive staining vessels expressed as a proportion of the total number of vessels in the superficial and mid-dermis of each section was used for analysis for each individual. (A) Representative images are shown of CD31 (green) and E-selectin (red) expression as well as merged views where overlap of the fluorochromes indicates activated endothelium. (B) Graph shows cumulative data and mean values for each group. Each symbol represents one individual. There are significantly fewer activated dermal vessels in the old individuals, both 1 and 3 days after VZV antigen challenge, compared to in the young (*p=0.016 and *p=0.027 respectively, Mann Whitney test).

4.4 Accumulation of cells of innate immune system at site of the DTH response

Cells of the innate immune response play a vital role in generating an adaptive immune response by virtue of functions such as pro-inflammatory cytokine production and antigen presentation to antigen-specific T cells. The inflammatory environment is particularly important for activating endothelium to enhance recruitment of leukocytes from the circulation. Accumulation of macrophages during the Mantoux test, a classical DTH responses has been well documented (Poulter, Seymour et al. 1982) and a variety of DC subsets are involved in cutaneous inflammation. We therefore hypothesised that a difference in these populations during the DTH response between the young and old volunteers may be contributing to impaired dermal activation and lack of T cell accumulation in the elderly. Many of the macrophage and DC markers overlap however it has been shown that CD163 can be used to selectively stain for macrophages whilst CD11c is a good selective marker for DCs in normal human skin (Zaba, Fuentes-Duculan et al. 2007).

4.4.1 Accumulation of dendritic cells at site of the DTH response

Immunohistochemical staining for CD11c expression was performed on skin sections from biopsies of normal skin or skin taken 1, 3, or 7 days after VZV antigen injection from old and young volunteers. Although similar numbers of $CD11c^+$ DCs were present in normal skin of both groups, there was a significant increase in numbers over the time course seen in the young, but not in the old (**p=0.008, Kruskal Wallis test) (Figure 4.11). By 24 hours post VZV antigen injection there were significantly more $CD11c^+$ DCs present in the young compared to the old (*p=0.040, Mann Whitney test).

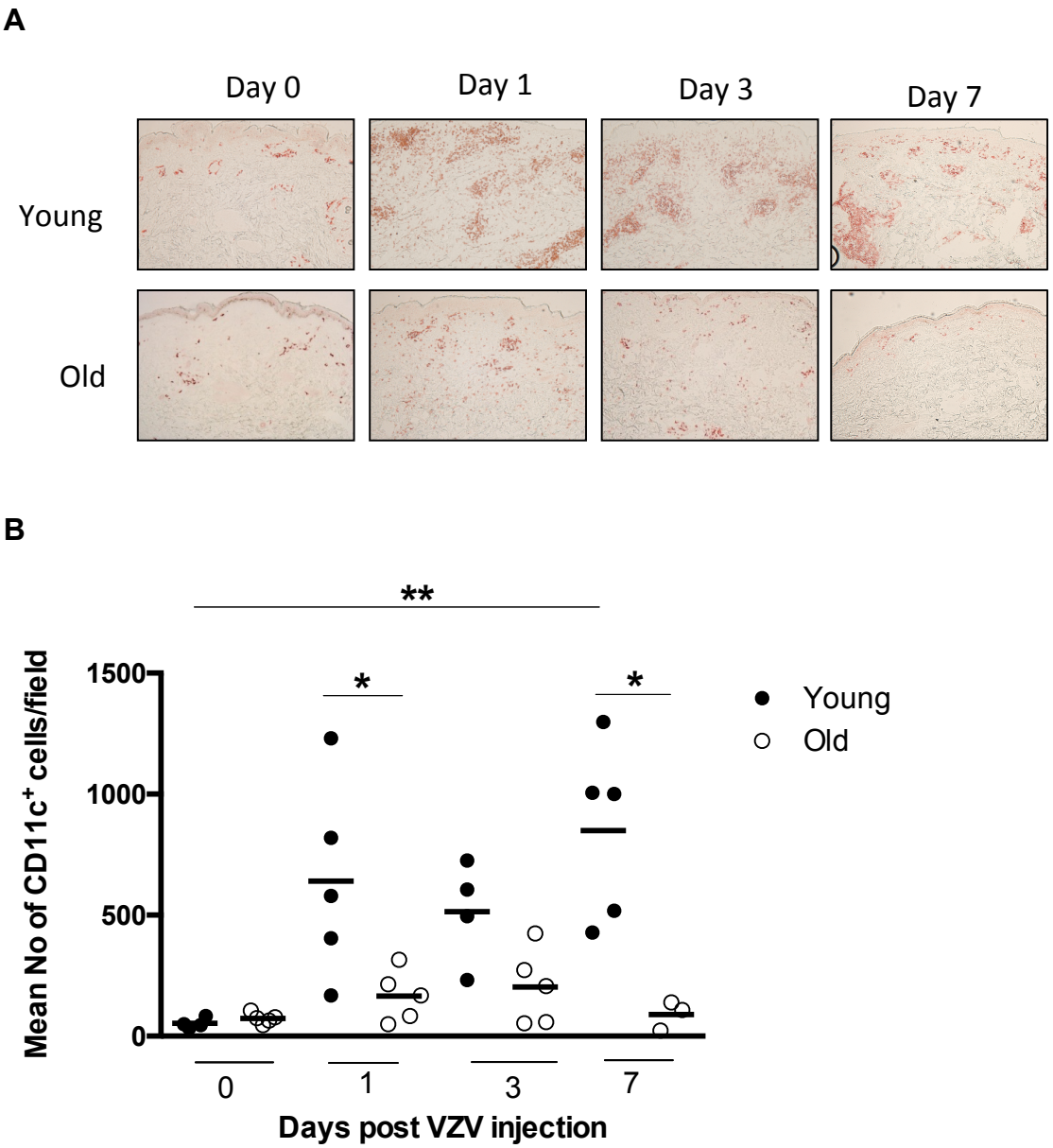


Figure 4.11 Accumulation of $CD11c^+$ dendritic cells at the site of cutaneous DTH response

Normal skin sections from old and young volunteers and those taken 1, 3 and 7 days after VZV antigen injection were stained for CD11c by immunohistochemistry (n=3-5 per age group at each time point). The number of positive cells was counted per field. Where more than one field was needed to assess the section, the mean number of cells per field was used for analysis. (A) Representative images are shown of old and young sections at all time points. (B) Graph shows cumulative data and the mean values for each group. Each symbol represents one individual. There is a significant increase in mean number of $CD11c^+$ DCs in the young over the time course (**p=0.008, Kruskal Wallis test) but not in the old. 24 hours after the skin test there were significantly more $CD11c^+$ DCs present in the young compared to the old (*p=0.040 Mann Whitney test) and this difference persisted at day 7 (*p=0.036, Mann Whitney test).

To determine whether this increase in $CD11c^+$ DCs in the young was made up of resident DCs or inflammatory dendritic cells recruited from the circulation into the skin, double immunofluorescence staining was performed to look for both CD11c and BDCA-1 expression. Resident $CD11c^+$ DCs are $BDCA\text{-}1^+$ while recruited inflammatory DCs e.g., Tip-DCs seen in psoriasis are not (Zaba, Krueger et al. 2009). The majority of $CD11c^+$ DCs in the skin in the young after the VZV antigen injection are $CD11c^+BDCA\text{-}1^-$ suggestive of an influx of inflammatory dendritic cells into the skin from the circulation (Figure 4.12).

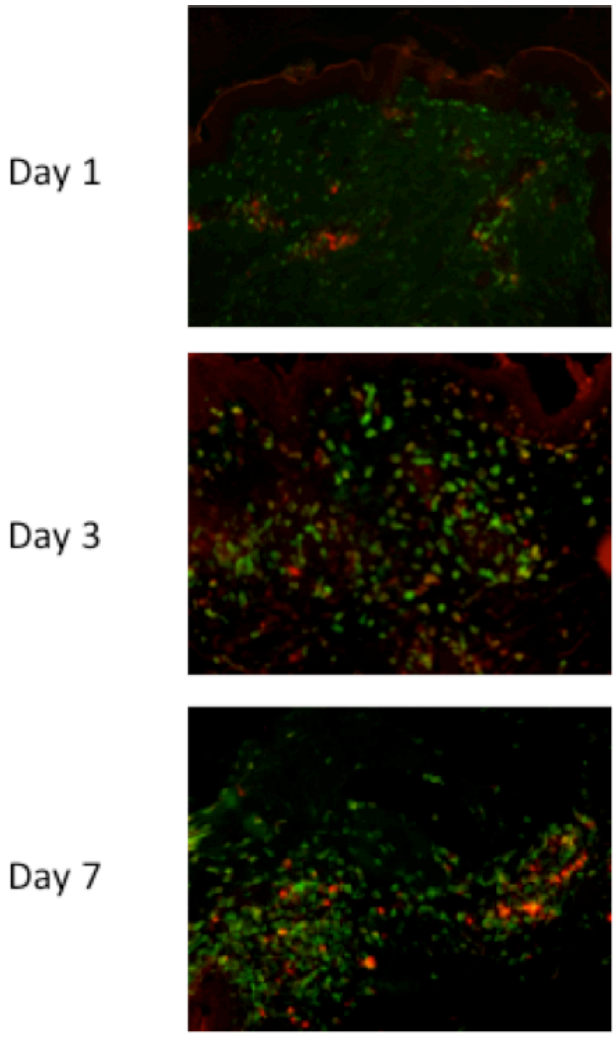


Figure 4.12 Phenotype of $CD11c^+$ dendritic cell infiltrate in young volunteers at site of cutaneous DTH response

Immunofluorescence staining was performed on skin sections from young volunteers taken 1,3 or 7 days after VZV antigen injection for expression of CD11c (green) and BDCA-1 (red). Representative photographs are shown for each time point after VZV

injection. The majority of $CD11c^+$ dendritic cells did not co-express BDCA-1 at either 24 hours or subsequent time points.

To further characterise these DCs we stained skin sections from old and young volunteers after VZV antigen injection for expression of dendritic cell lysosome-associated membrane protein (DC-LAMP). DC-LAMP is a marker of dendritic cell maturation and is thought to play a role in the trafficking of MHC-antigen complexes (de Saint-Vis, Vincent et al. 1998). DC-LAMP expression increases significantly in the young over the time course (days 1 to 3 *p=0.03, Mann Whitney test, and days 1 to 7 **p=0.007, Kruskal Wallis test) resulting in significantly more mature DCs in the young compared to the old by day 7 post VZV antigen injection (*p= 0.03, Mann Whitney test) (Figure 4.13). This suggests that a proportion of the DCs present underwent maturation in the skin in young volunteers during the DTH response.

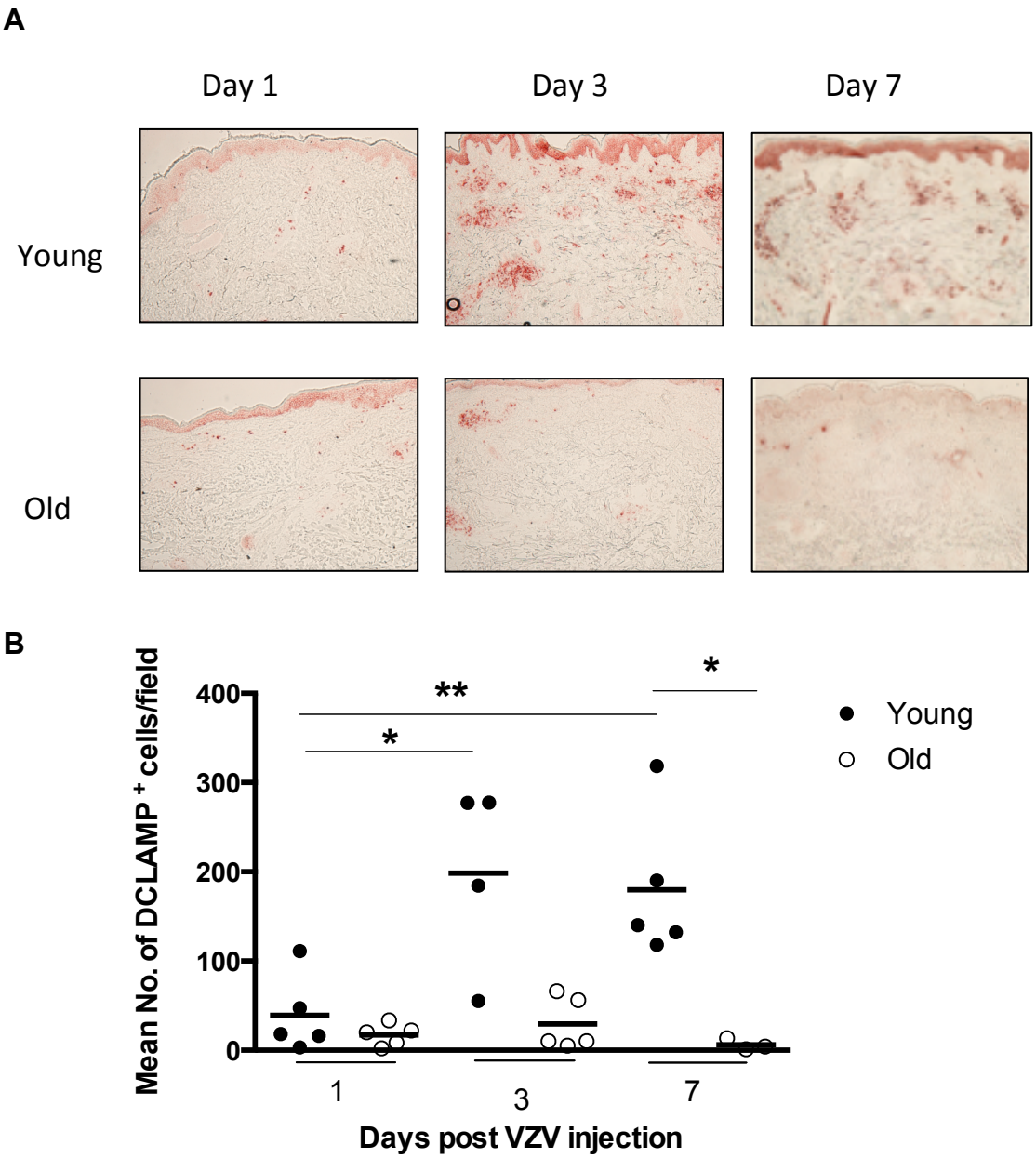


Figure 4.13 DC-LAMP expression at the site of DTH response

Skin sections taken 1, 3 or 7 days after VZV antigen injection from old and young volunteers (n=3-5 per age group at each time point) were stained for DC-LAMP expression by immunohistochemistry. The number of positive cells was counted per field. Where more than one field was needed to assess the section, the mean number of cells per field was used for analysis. (A) Representative images of DC-LAMP expression in sections from old and young volunteers at all time points after VZV antigen injection are shown. (B) Graph shows cumulative data and mean for each group. Each symbol represents one individual. Mean DC-LAMP expression significantly increases in young but not old individuals over the time course (**p=0.007, Kruskal Wallis test). There is a statistically significant increase in the mean expression of DC-LAMP in the young cohort between days 1 and 3 (*p=0.03, Mann Whitney test), and a significant difference in mean expression of DC-LAMP between the young and old by day 7 (*p=0.03, Mann Whitney test).

Plasmacytoid dendritic cells (pDCs) are a type of circulating dendritic cell, characterised by their ability to produce high quantities of type 1 interferons during viral infections (Gilliet, Cao et al. 2008). They are not found in significant numbers in normal skin and they do not express CD11c but do exclusively express BDCA-2 (Dzionek, Fuchs et al. 2000). Since pDCs are known to accumulate rapidly in inflamed tissues (Nestle, Conrad et al. 2005) and given the viral nature of our antigen, we sought to determine whether there was any difference in the presence of these cells in the skin of old and young volunteers before or after VZV antigen injection. pDCs were identified by performing immunohistochemistry for BDCA-2 expression on the relevant skin sections. A small number of pDCs were seen in the skin of young volunteers after VZV antigen injection with an increasing trend over the time course but not reaching statistical significance by day 3 ($p=0.056$, Kruskal Wallis test, Figure 4.14). Day 7 data is not shown due to an inadequate number (<3) of data points. A negligible number of pDCs were seen in the skin of old volunteers both before and after VZV antigen injection.

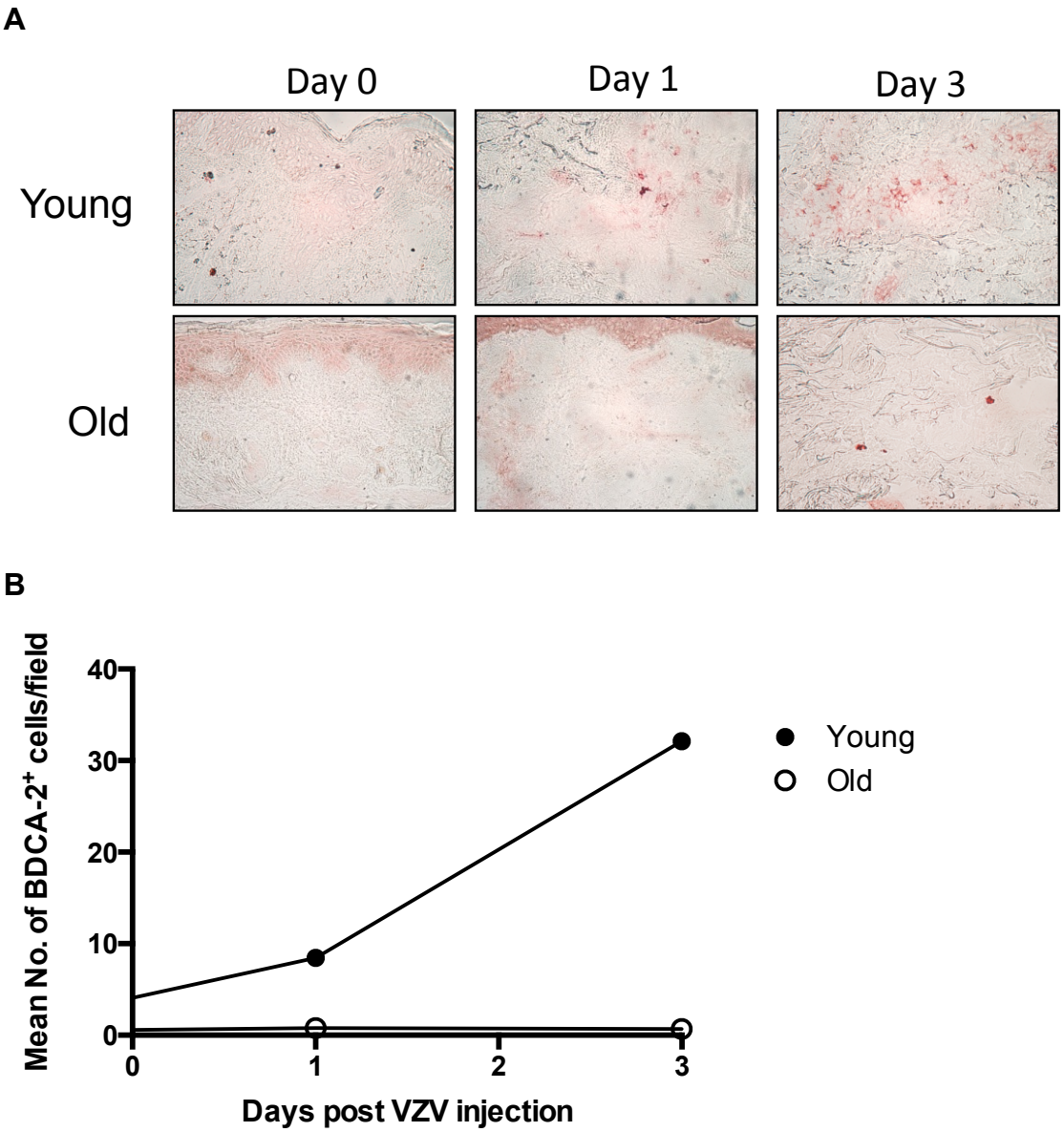


Figure 4.14 Presence of plasmacytoid dendritic cells at the site of the DTH response

Skin sections from biopsies of normal skin or 1 or 3 days after VZV antigen injection from old and young volunteers (n=3-4 per age group at each time point) were stained for BDCA-2 expression by immunohistochemistry. The number of positive cells was counted per field. Where more than one field was needed to assess the section, the mean number of cells per field was used for analysis. (A) Representative images of BDCA-2 staining of sections from young and old volunteers are shown. (B) Graph shows cumulative data and the mean value for each group. There is an increase in the mean number of PDCs seen in the young between day 0 and day 3 although this did not reach statistical significance (p=0.056, Kruskal Wallis test).

pDCs may play a role in the DTH response to VZV antigen alongside the influx of the much larger $CD11c^+$ DC population in the skin of young people.

4.4.2 Accumulation of macrophages at site of the DTH response

Skin sections from biopsies of normal skin and at 1, 3 or 7 days after VZV antigen injection in old and young volunteers were stained for CD163 expression by immunohistochemistry. Although in normal skin and 1 day after VZV antigen injection, macrophage numbers were comparable in the two groups, there was a significant increase in the number of macrophages in the young volunteers over the time course not seen in the old (**p=0.006, Kruskal Wallis test) (Figure 4.15). By day 3, the mean number of macrophages peaked in the young. There were significantly more macrophages present in the skin of young volunteers compared to old at day 7 (*p=0.033, Mann Whitney test).

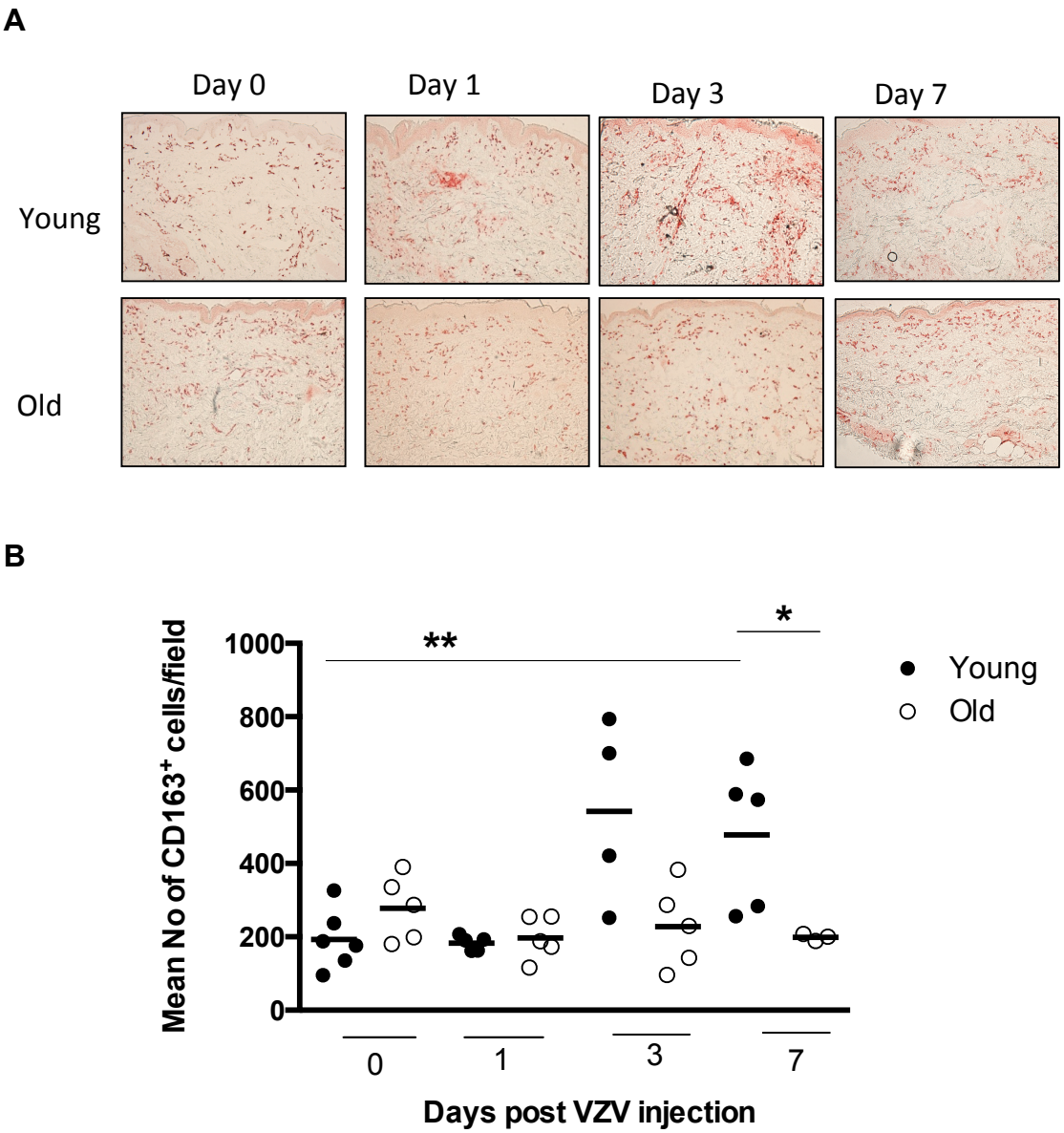


Figure 4.15 Accumulation of CD163$^+$ macrophages at the site of cutaneous DTH response

Sections of normal skin and skin taken 1,3 or 7 days post VZV antigen injection from old and young volunteers (n=3-6 per age group at each time point) were stained for CD163 expression by immunohistochemistry. The number of positive cells was counted per field. Where more than one field was needed to assess the section, the mean number of cells per field was used for analysis. (A) Representative images of CD163$^+$ cells are shown in old and young skin sections at the stated time points. (B) Graph shows cumulative data and the mean value for each group. Each symbol represents one individual. There is a significant increase in the mean number of CD163$^+$ macrophages per field infiltrating the skin in young volunteers over the time course (**p=0.006, Kruskal Wallis test) not evident in the old. By day 7 there are significantly more CD163$^+$ macrophages present per field in the young compared to old skin (*p=0.033, Mann Whitney test).

4.5 Production of TNF-α at the site of DTH response

In previous work investigating the cutaneous DTH response to *C. albicans* antigen, our group showed that decreased TNF-α production by macrophages was associated with a reduced delayed type hypersensitivity response in the elderly (Agius, Lacy et al. 2009). In that study, in contrast to this work, there were similar numbers of both macrophages and DCs in young and old skin during the DTH response. TNF-α, produced by macrophages and dendritic cells may play a pivotal role in the DTH response by activating dermal endothelium. In light of there being larger numbers of both macrophages and $CD11c^+$ DCs in the DTH response to VZV antigen in the young individuals compared to the old, we would expect there to also be more TNF-α. However, we were interested in confirming which of these innate cells was the predominant cell source for TNF-α production in the current scenario given the early influx of DCs. Skin sections from biopsies taken 1 and 3 days after VZV skin test antigen from old and young volunteers were stained for TNF-α and either CD11c or CD163 expression by immunofluorescence (Figure 4.16). As expected, there is more TNF-α present in the skin sections from young skin compared to old, and staining is brightest in a perivascular distribution. However, it is difficult to accurately determine which cell in the perivascular infiltrate, T lymphocytes, macrophages or dendritic cells, is the predominant source of the pro-inflammatory cytokine.

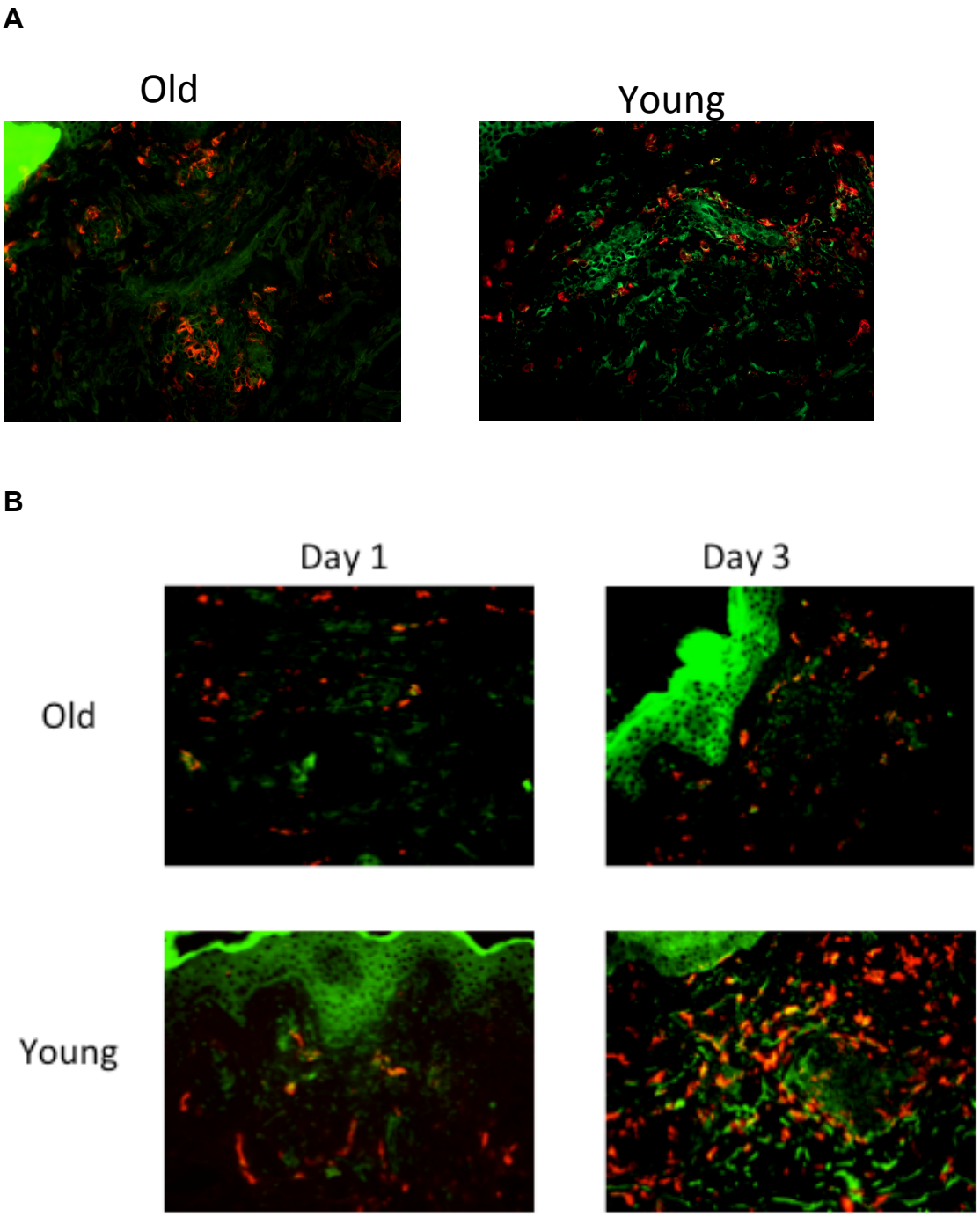


Figure 4.16 TNFα expression at the site of DTH response

Skin sections from old and young volunteers taken after VZV skin test were co-stained by immunofluorescence for TNFα and either CD11c or CD163 expression. (A) Representative images of a skin section from an old and young volunteer taken 1 day after VZV antigen injection stained for CD11c (red) and TNFα (green) expression. (B) Representative images of old and young skin sections taken at 1 and 3 days after VZV antigen injection, showing CD163 (red) and TNFα (green) expression.

4.6 Early events at the site of the DTH response

The DTH response is an antigen-specific memory T cell response and we have shown that the function of circulating antigen-specific T cells in response to *in vitro* stimulation, as well as the *in vivo* accumulation of antigen-specific T cells at the site of the DTH response is impaired in the elderly. There is also reduced accumulation of dendritic cells and reduced activation of dermal endothelium at 24 hours in the elderly and these factors could hinder recruitment of circulating leukocytes. Next we investigated whether differences in the DTH response between the two age groups were apparent even before 24 hours. Specifically we investigated activation of dermal endothelium, neutrophil infiltration, and $CD4^+$ T cell and dendritic cell accumulation 6 hours after VZV antigen injection. For these experiments, volunteers were also injected with normal saline intra-dermally on the contralateral arm to provide an internal control for changes related to trauma of the injection alone.

4.6.1 Activation of dermal endothelium early in the DTH response

We stained skin sections from biopsies taken from old and young volunteers 6 hours after intradermal VZV antigen injection in one forearm and normal saline injection in the other for expression of CD31 and E-selectin by immunofluorescence. There was no significant difference in the expression of E-selectin 6 hours after either VZV or normal saline injection between old and young individuals with approximately 25-30% of vessels expressing E-selectin (Figure 4.17). This suggests that there is a defect in the subsequent ability to amplify this level of activation during an antigen specific response in the elderly compared to that seen in young individuals at 24 hours where approximately 75% of dermal vessels are activated (Figure 4.10).

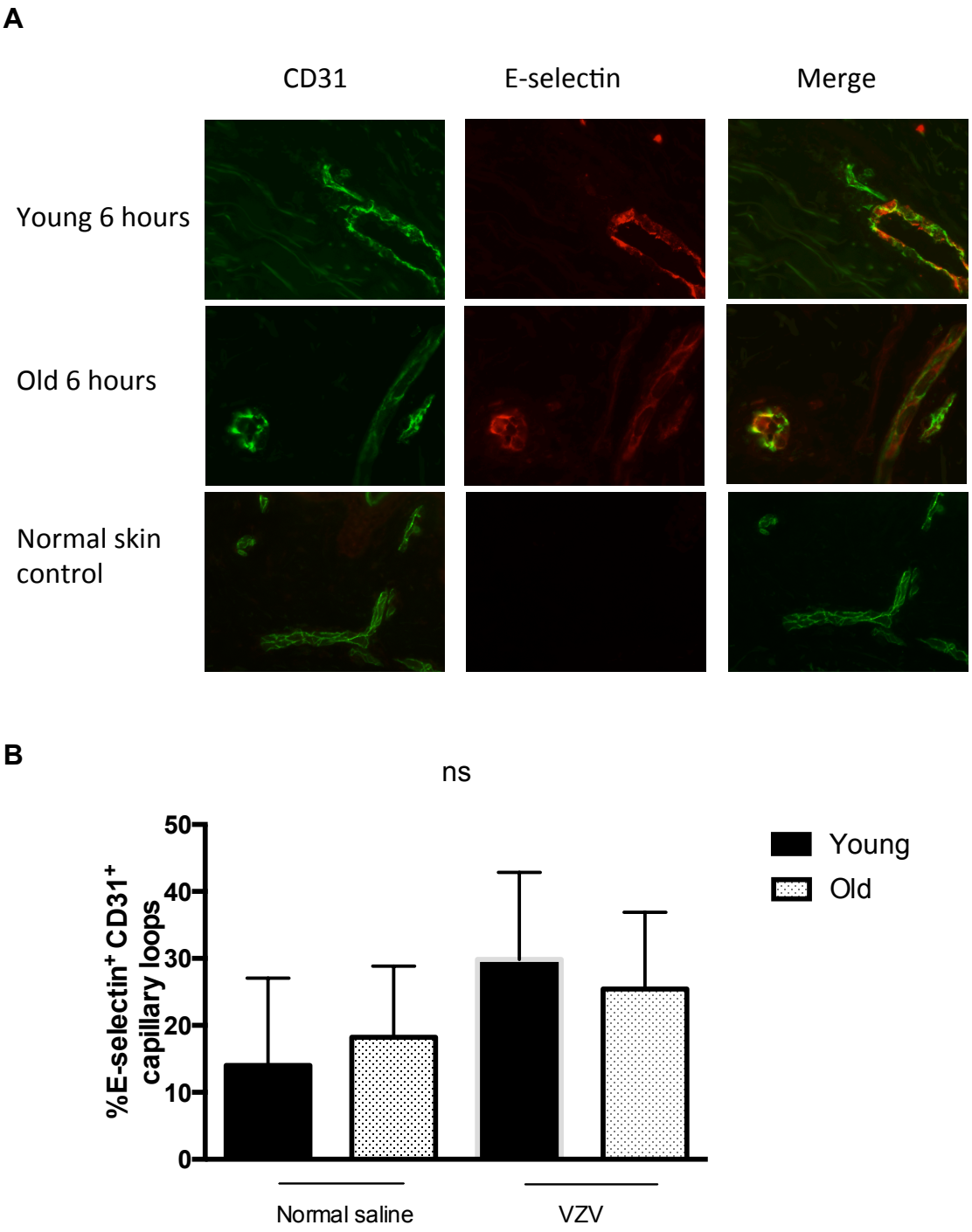


Figure 4.17 Activation of dermal endothelium early in cutaneous DTH response

Skin sections from biopsies taken 6 hours after intradermal injection of 0.02ml of VZV antigen into one proximal forearm and 0.02ml of normal saline injection into the other from old (n=5) and young (n=5) volunteers were stained for CD31 and E-selectin expression by immunofluorescence. The percentage of double staining vessels in the superficial and mid-dermis were counted per section for each individual and used for analysis. (A) Representative images of VZV injected skin are shown for old and young volunteers: CD31 (green), E-selectin (red), with overlapping images showing

activated endothelium. (B) Graph shows mean and standard deviation of cumulative data for each group. No significant difference was found in the expression of E-selectin between the age groups following either VZV antigen or normal saline injection (Mann Whitney and Wilcoxon tests).

4.6.2 Accumulation of neutrophils early in the DTH response

Neutrophils are typically the first leukocyte population recruited from the circulation into inflamed tissue under the influence of chemokines and activated endothelium. Given that dermal endothelium is equally activated in old and young volunteers early in the DTH response (6 hours post VZV antigen injection) we hypothesised that neutrophils should accumulate equally in the old and young. To determine whether neutrophil recruitment was impaired, we stained skin sections from biopsies taken from old and young volunteers 6 hours after intradermal VZV antigen injection in one forearm and normal saline injection in the other for expression of neutrophil elastase expression by immunohistochemistry. Similar numbers of neutrophils were recruited to the DTH site in old and young individuals 6 hours after VZV antigen and normal saline injection (Figure 4.18).

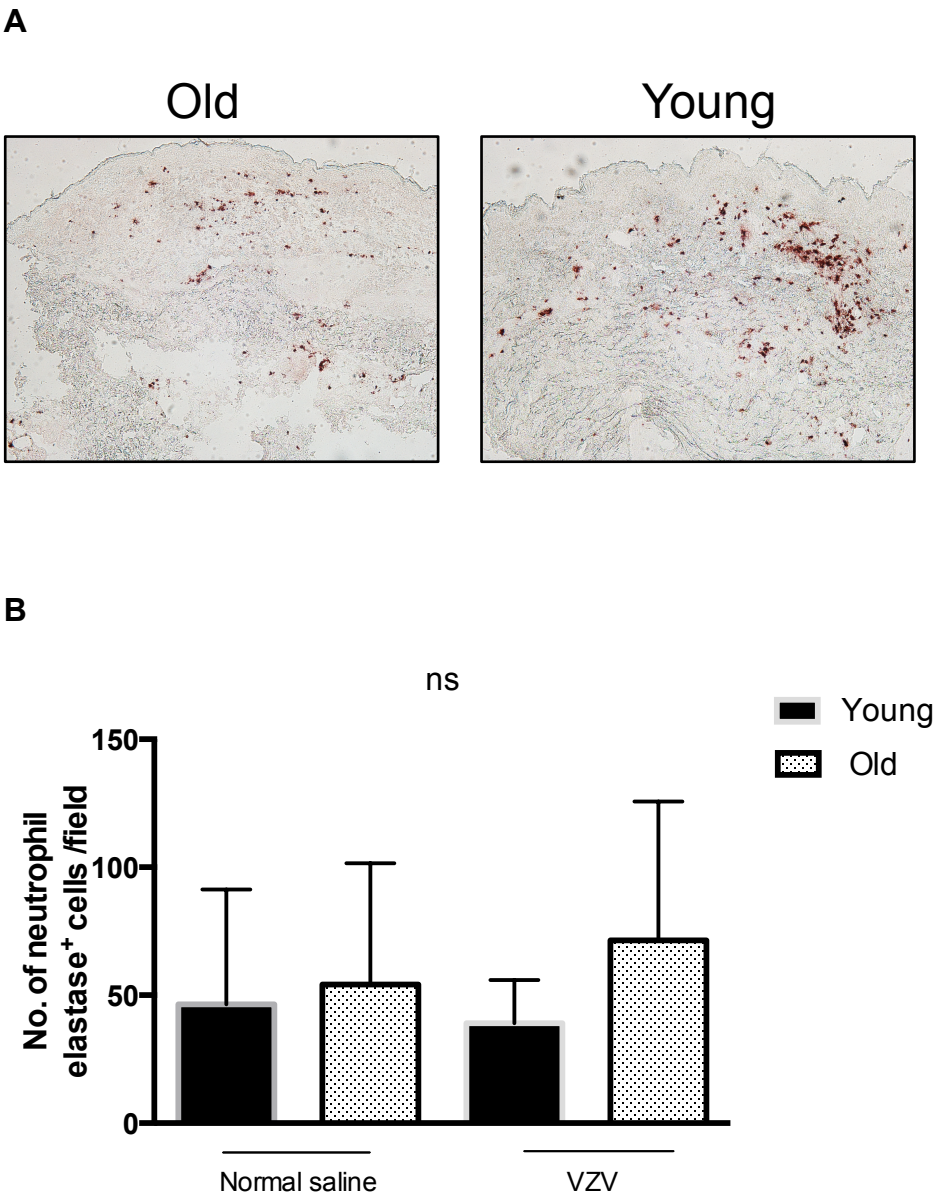


Figure 4.18 Accumulation of neutrophils early in cutaneous DTH response

Skin sections from biopsies taken 6 hours after intradermal injection of 0.02ml of VZV antigen into one proximal formarm and 0.02ml of normal saline injection into the other from old (n=5) and young (n=5) volunteers were stained for neutrophil elastase expression by immunohistochemistry. (A) Representative images of staining for neutrophil elastase expression in VZV injected skin are shown for old and young volunteers. (B) Graph shows mean of cumulative data and standard deviation for each group. No difference in the number of infiltrating neutrophils between old and young volunteers in skin injected with either VZV antigen or normal saline was found (Mann Whitney and Wilcoxon tests).

4.6.3 $CD4^+$ T cell accumulation early in the DTH response

To determine whether $CD4^+$ T cell accumulation is evident as early as 6 hours after antigen challenge, we stained normal skin sections and sections from old and young volunteers 6 hours after intradermal VZV skin test injection in one forearm and normal saline injection in the other for CD4 expression by immunohistochemistry. There was no significant difference in the numbers of $CD4^+$ T cells present in normal or injected skin between the two age groups (Figure 4.19). We were not able to detect a statistically significant increase in the number of $CD4^+$ T cells over the 6 hours in the young volunteers.

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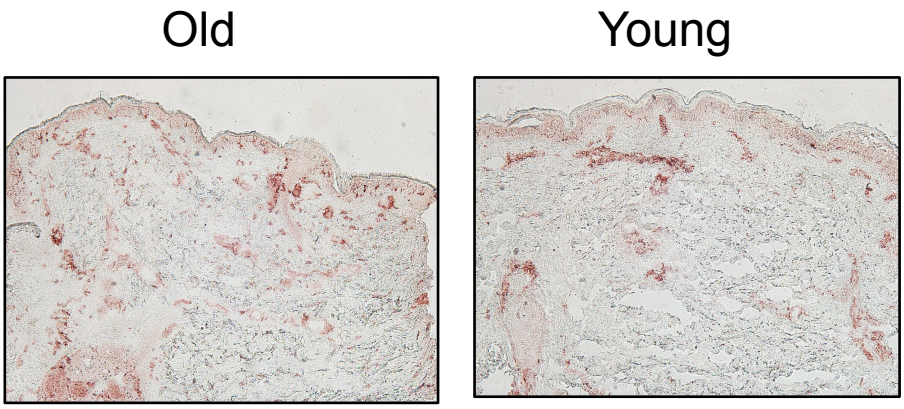

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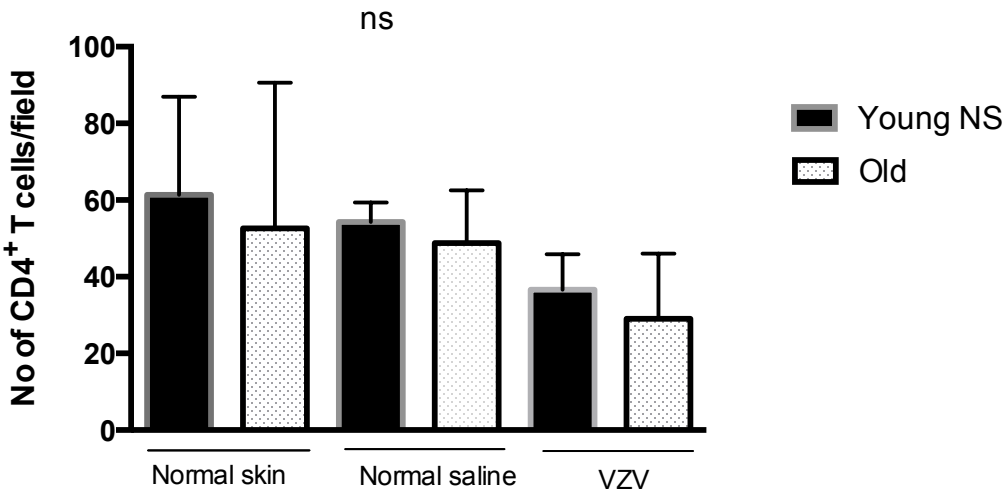

Figure 4.19 Accumulation of $CD4^+$ T cells early in cutaneous DTH response

Skin sections from biopsies of normal skin and from skin taken 6 hours after intradermal injection of 0.02ml of VZV antigen into one proximal formarm and 0.02ml of normal saline injection into the other from old (n=5) and young (n=5) volunteers were stained for CD4 expression by immunohistochemistry. (A) Representative images of staining for CD4 expression in VZV injected skin are shown for old and young volunteers. (B) Graph shows mean of cumulative data and standard deviation for each group. No statistically significant difference in the number of infiltrating $CD4^+$ T cells was found between old and young volunteers in normal or injected skin, nor was there a significant increase in numbers between baseline and 6 hours after injection in either age group (Mann Whitney and Wilcoxon tests).

4.6.4 Accumulation of dendritic cells early in the DTH response

We have shown that the number of $CD11c^+$ dendritic cells in the skin significantly increases in young compared to old volunteers by 24 hours after VZV antigen injection. To determine if this trend can be detected early in the DTH response, we stained skin sections from old and young volunteers 6 hours after intradermal VZV skin test injection in one forearm and normal saline injection in the other for CD11c expression by immunohistochemistry. Six hours after VZV skin test antigen injection the number of $CD11c^+$ dendritic cells increases significantly in the young (*p=0.03, Mann Whitney test) (Figure 4.20). Despite this there is no statistically significant difference in the number of $CD11c^+$ DCs between the young and old 6 hours after VZV injection and this only becomes evident at 24 hours (Figure 4.11). However, in the majority of old volunteers (n=4 out of 5) the number of $CD11c^+$ cells is lower than that seen in the young 6 hours after injection with VZV antigen suggestive of a trend. The number of $CD11c^+$ cells present 6 hours after injection with normal saline was not significantly different from those seen after VZV antigen injection. The sections were also stained for expression of Ki67 and CD11c by immunofluorescence but cells co-expressing the markers, indicative of local proliferation, were rare in both age groups. While some of the dendritic cells may have migrated from distal parts of the skin to the site of antigen challenge, our data supports an influx of dendritic cells in the young from the circulation (Figure 4.12).

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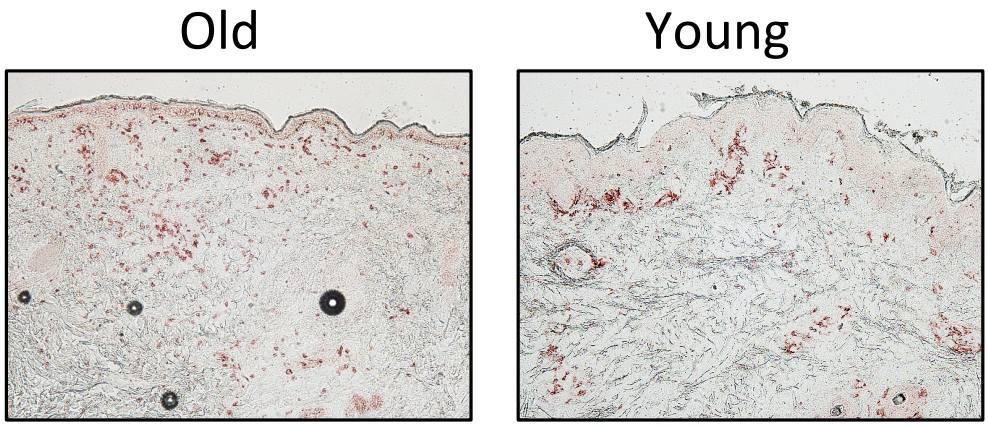

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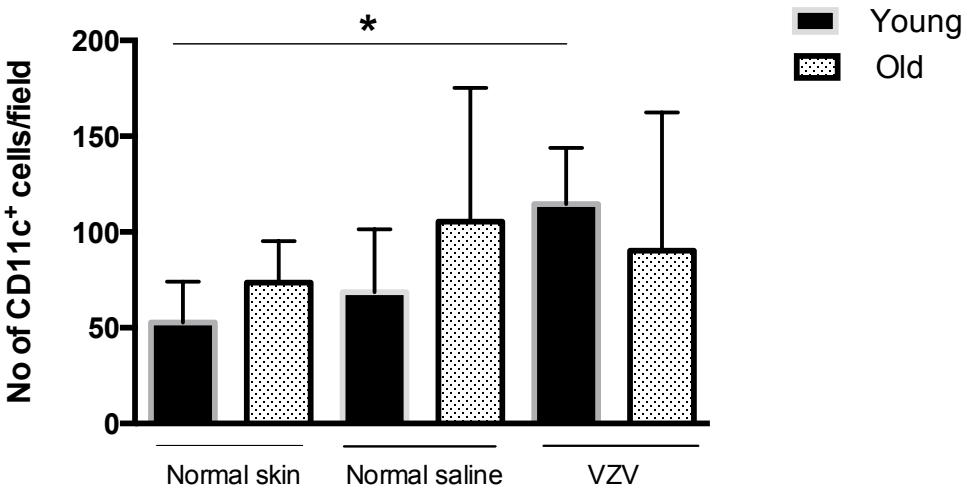


Figure 4.20 CD11c$^+$ dendritic cell presence early in cutaneous DTH response

Skin sections from biopsies taken of normal skin from old (n=5) and young (n=4) volunteers, and 6 hours after intradermal injection of 0.02ml of VZV antigen into one proximal forearm and 0.02ml of normal saline into the other from old (n=5) and young (n=5) volunteers were stained for CD11c$^+$ expression by immunohistochemistry. (A) Representative images of staining for CD11c expression in VZV injected skin are shown for old and young volunteers. (B) Graph shows mean of cumulative data and standard deviation for each group. There is a significant increase in the number of infiltrating CD11c$^+$ cells in the young 6 hours after the injection of VZV (*p=0.03 Mann-Whitney test) but not in the old (p=0.67 Mann-Whitney test) nor in either age group after injection with normal saline. There was no significant difference in the number of CD11c$^+$ cells between young and old in either normal skin or after normal saline or VZV injection (Mann Whitney test). Nor was there any difference in the number of CD11c$^+$ cells present within the same age group after VZV or normal saline injection (Wilcoxon matched-pairs test).

4.7 Discussion

In this chapter we report that that old individuals have an impaired cutaneous DTH response to VZV antigen manifest by an absence of the T cell accumulation that occurs in the young. Old and young individuals have similar numbers of T cells in normal skin however the trend for a subsequent increase in number is evident by 24 hours after VZV injection in young individuals only. By day 3 the number of T cells is significantly higher in the young compared to the old individuals with the greatest number of T cells seen at day 7. This accumulation is in part due to local proliferation of T cells in the skin. Interestingly the peak in T cell accumulation occurs after the peak clinical response rather than coinciding with it. However, the clinical score does correlate with the number of infiltrating T cells suggesting that the early inflammatory response is crucial to the development of subsequent T cell accumulation.

It is possible that in the elderly the lack of T cell accumulation reflects a reduced ability of antigen specific T cells to migrate into or proliferate in the skin in response to antigen. However, we propose that the impaired T cell response may reflect a failure of other cells present in the skin e.g., dendritic cells, macrophages or resident T cells to condition the cutaneous environment adequately to allow T cell recruitment and/or proliferation.

Cells resident in normal skin are required to initiate the DTH response and an early feature of inflammation is activation of the dermal endothelium. Activated endothelium up regulates expression of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 to recruit circulating leukocytes including T lymphocytes (Bevilacqua 1993). Previous work has shown that expression of E-selectin was reduced in cutaneous squamous cell carcinomas and that these contained few CLA^+ T cells. Treatment with imiquimod, a TLR 7 agonist, up-regulated E-selectin expression and was associated with an influx of CLA^+ T cells (Clark, Huang et al. 2008). We found that in the elderly despite similar low-level expression of E-selectin at 6 hours in both groups

after either VZV antigen or normal saline injection, there is a subsequent failure to amplify this in the elderly after VZV antigen injection. Twenty-four hours after VZV injection, 70% of dermal vessels express E-selectin in the young individuals but only 30% of vessels do so in the elderly and this may contribute to reduced recruitment of T cells in the old. It would be of interest to investigate the expression of other adhesion molecules such as ICAM-1 and VCAM-1 to see if they are similarly reduced in the elderly, as was shown in the DTH response to *Candida albicans* (Agius, Lacy et al. 2009).

Pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1α are known to activate dermal endothelium (Lee, Chung et al. 1995). Dendritic cells, macrophages and $CD4^+$ T_{H1} cells may act as early producers of these cytokines. Therefore it may be that these skin resident innate cells are defective in the elderly. Alternatively tissue resident antigen specific T cell populations have recently been identified (Gebhardt, Whitney et al. 2011; Teijaro, Turner et al. 2011; Jiang, Clark et al. 2012) and it may be that VZV-specific T cells present in the skin may be reduced in number or function in the elderly with reduced production of pro-inflammatory cytokines from these cells. Of note, while TNF-α and IL-1α induced expression of E-selectin peaks after 6 hours, IFN-γ induced expression of E-selectin on dermal endothelium is not detectable until 24 hours and peaks at 48 hours (Lee, Chung et al. 1995). Alternatively or in addition to the above, the function of any of these skin resident populations may be suppressed by skin resident Tregs (Takahashi, Kuniyasu et al. 1998). Clinically Tregs in squamous cell carcinomas of the skin can make up 50% of the infiltrating T cell population and effective treatment is associated with both reducing Treg number and function (Clark, Huang et al. 2008). In support of a prominent role for Tregs in influencing T cell accumulation, we found that Tregs are present in greater proportions in elderly individuals with impaired DTH responses compared to both young individuals and the small number of elderly volunteers who maintain relatively robust clinical responses.

In addition to T cells, other circulating populations are also recruited to the skin during a DTH response including neutrophils, DCs and macrophages. Recruitment of these cells may be affected by an inadequate production of pro-inflammatory cytokines or chemokines early in the DTH response, and/or these recruited populations may influence subsequent T cell recruitment. Neutrophils are typically the first leukocytes to be recruited during an inflammatory response and were present in similar numbers in the skin of both old and young individuals 6 hours after VZV antigen or normal saline injection. This is likely to represent an antigen non-specific response and is seen after needle-induced tissue damage (Peters, Egen et al. 2008). Although there is evidence that particular neutrophils subsets are able to influence the subsequent immune response (Tsuda, Takahashi et al. 2004), murine studies where neutrophils have been depleted demonstrate that while the acute phase of the DTH response is suppressed, late phase T cell infiltration is not significantly affected (Hwang, Yamanouchi et al. 2004; Doebis, Siegmund et al. 2008).

Six hours after VZV antigen injection we found that $CD11c^+$ dermal dendritic cells start to accumulate in the young but not the elderly. This accumulation is not seen after injection of normal saline in young individuals suggesting that it is an antigen specific response. The number of $CD11c^+$ DCs is significantly greater in the young individuals than in the old by 24 hours. The lack of BDCA-1 and Ki67 expression on the majority of these cells leads us to conclude that these are most likely a recruited population from the circulation. The increase in expression of DC-LAMP between day 1 and day 3 suggests these DCs are undergoing maturation in the skin (de Saint-Vis, Vincent et al. 1998). In this state they would be ready to activate antigen specific T cells in the skin and contribute to amplifying the specific immune response. A recent study in humans has shown that the level of DC recruitment correlated with the frequencies of T cells infiltrating the DTH site (Liang, Bond et al. 2013). However, It is not clear in our work whether the DCs recruited in the early phase of the response influence early T cell recruitment, or whether both populations are responding to antigen specific signals from skin resident cells.

Little is known about recruitment of inflammatory DC populations into the skin, but is likely to involve specific chemokines. A DC-specific C-type lectin, DC-SIGN interacts with ICAM-2 to mediate chemokine-induced migration across both resting and activated endothelium (Geijtenbeek, Krooshoop et al. 2000) and it would be of interest to compare expression of DC-SIGN on circulating DC populations in old and young individuals. Although E-selectin may also mediate DC migration along activated endothelium (Srinivas, Larsson et al. 1993), there was no difference in expression of E-selectin between the young and the elderly at the time of earliest DC recruitment in the young to account for the difference.

Macrophages are also present in normal skin and like DCs, are early producers of pro-inflammatory cytokines and upon activation may present antigen to $CD4^+$ T cells. There is a similar number of macrophages present in the normal skin of young and old individuals. An increase in the number of macrophages is seen 48 hours after VZV antigen injection but only in young individuals. At this late time point, these recruited cells are unlikely to play a significant role in influencing the early antigen-specific response although they may subsequently contribute to amplification of the antigen-specific T cell response. However, as mentioned above there may be an age-related defect in the function of those macrophages present in normal skin required to initiate the memory response, reduced activation of macrophages, or increased suppression by Tregs in the elderly.

We propose that the main reason for the reduced accumulation of T cells in the elderly in response to VZV antigen is inadequate conditioning of the cutaneous environment to support recruitment of antigen-specific T cells. This may be as a result of a reduced number of functioning resident antigen-specific T cells and/or innate cells which fail to stimulate an adequate early immune response. The increased proportion of Tregs in the skin of the elderly prior to antigen challenge is in keeping with an increased potential to suppress local T cell responses.

In the young individuals we found that $CD4^+Foxp3^+$ cells accumulate over the time course after VZV injection in line with the corresponding $CD4^+$ effector T cell population presumably to regulate the immune response. We hypothesised that these two populations would share antigen specificity and went on to investigate this in the next chapter.

Chapter 5: Detection of VZV-specific $CD4^+$ T cells and characterisation of $CD4^+Foxp3^+$ regulatory T cells that accumulate during the cellular response to VZV antigen in the skin

5.1 Introduction

In the last chapter we identified that $CD4^+$ and $CD4^+Foxp3^+$ T cells accumulate at the site of antigen challenge in the young. We also showed that the proportion of Tregs typically increases in the skin with age and that this is associated with a reduced clinical and effector T cell response to VZV antigen injection in old volunteers. In this chapter we investigate whether the $CD4^+$ T cells seen in the skin after VZV antigen challenge are specific for VZV. We also further characterise the $CD4^+Foxp3^+$ T cells to determine whether they are consistent with Tregs and share antigen specificity with the effector T cell population i.e. VZV-specific Tregs. Tregs are a heterogenous population made up of nTregs, produced by the thymus, and iTregs, which may arise extrathymically from naïve (Azukizawa, Dohler et al. 2011; Zhou, Horai et al. 2012) or memory T cells (Walker, Carson et al. 2005; Vukmanovic-Stejic, Agius et al. 2008; Huang, Dawicki et al. 2010; Schwele, Fischer et al. 2012). .

To further characterise the leucocytes involved in the recall response to VZV antigen in the skin, we induced suction blisters over the site of antigen injection from which leucocytes were subsequently isolated and analysed for markers of interest by flow cytometry (Akbar, Reed et al. 2013). In particular we used a flurochrome-conjugated peptide-MHC tetramer to identify VZV-specific T-cells, within both effector and Treg subsets. Peptide-MHC class II tetramers display an antigenic peptide in the class II-binding groove and in this way act as a surrogate for the interaction that occurs between APCs and antigen specific T cells (Nepom 2012). The tetramer was used to detect intermediate early 63 (IE63) - specific $CD4^+$ T cells. IE63 is a viral tegument

protein (Kinchington, Bookey et al. 1995) expressed during active VZV infection in the skin (Debrus, Sadzot-Delvaux et al. 1995; Nikkels, Debrus et al. 1995) and is present in ganglionic neurons during latency (Mahalingam, Wellish et al. 1996). Since old volunteers did not mount a significant clinical response to VZV injection, this work was performed in young volunteers in whom, although the Treg population as a proportion of total $CD4^+$ T cells was smaller, the actual number was higher, and therefore more suitable for further study.

5.2 Accumulation of VZV-specific $CD4^+$ T cells in the skin after VZV antigen injection

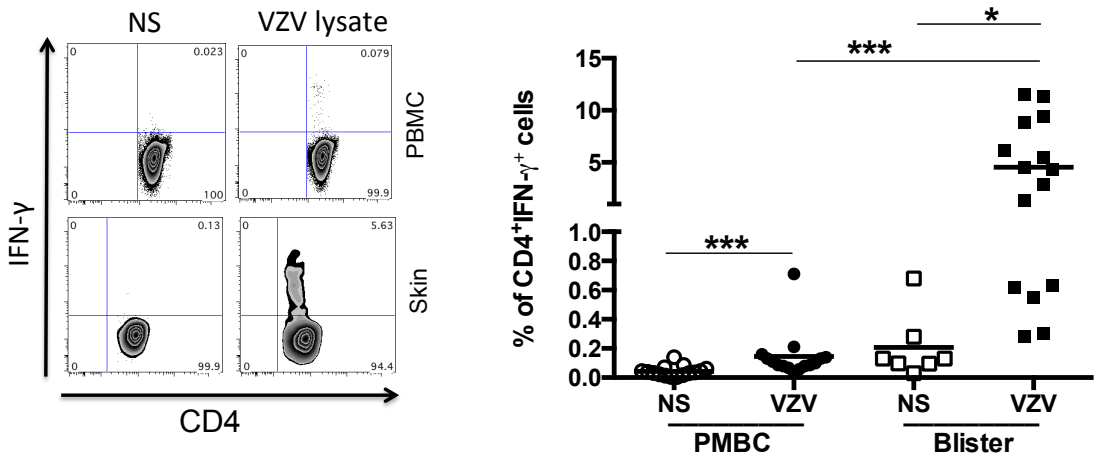
To identify VZV specific $CD4^+$ T cells we first induced a DTH response in young volunteers with a history of chickenpox by injecting 0.02ml VZV antigen intra-dermally into the medial aspect of the proximal forearm. Six days after the initial injection, we raised a skin suction blister over the site of the clinical response. The following day i.e. 7 days after VZV injection, coinciding with the peak cellular infiltrate (Figure 4.2), blister fluid was aspirated and a peripheral blood sample was taken from the volunteer. Blister cells and PBMCs were used for subsequent experiments. VZV-specific $CD4^+$ T cells were identified using either an HLA-DRB1*1501 restricted IE63 tetramer or by their ability to synthesise IFN-γ or IL-2 after overnight *in vitro* re-stimulation with VZV lysate.

5.2.1 Assessment of the frequency of VZV specific $CD4^+$ T cells by intracellular cytokine staining

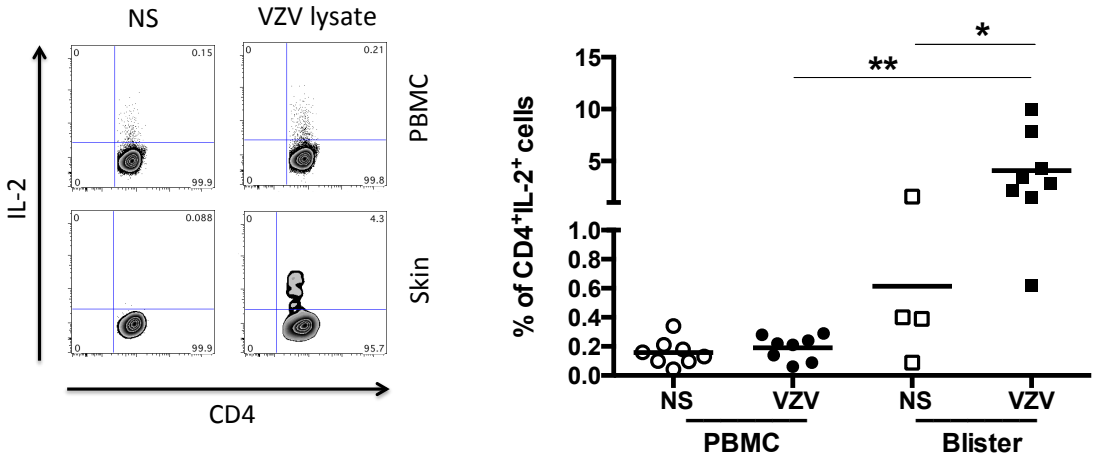
PBMCs and blister cells were isolated 7 days after cutaneous VZV antigen injection and stimulated for 15 hours with VZV lysate in the presence of Brefeldin A. Non-stimulated controls were also set up providing there was adequate numbers of blister cells isolated from the blister fluid. Cells were stained for the intracellular expression of IFN-γ or IL-2 and analysed by flow

cytometry. We found a significantly higher proportion of IFN-γ$^+$ CD4$^+$ VZV-specific T cells and IL-2$^+$ CD4$^+$ VZV-specific T cells present in blister fluid compared to peripheral blood (***p=0.0002 and **0.008 respectively, Wilcoxon paired test) (Figure 5.1A, B). While there were negligible IFN-γ$^+$ or IL-2$^+$ VZV-specific CD4$^+$ T cells in the non-stimulated peripheral blood sample, significantly higher levels were produced in the non re-stimulated blister sample, most probably reflecting cytokine production by VZV specific CD4$^+$ T cells that were activated *in situ* in the skin in response to the VZV antigen injection (*p=0.014 pooled data for IFN-γ and IL-2, Wilcoxon paired test). A large proportion of VZV specific CD4$^+$ T cells produced both IFN-γ and IL-2 (mean 44.3%) (Figure 5.1C).

A


NS
VZV lysate
PBMC
Skin
IFN-γ
CD4
0.023
0.079
100
99.9
0.13
99.9
5.63
94.4
% of CD4+IFN-γ+ cells
***
***
*
NS
VZV
NS
VZV
PMBC
Blister

B


NS
VZV lysate
PBMC
Skin
IL-2
CD4
0.15
99.9
0.21
99.8
0.088
99.9
4.3
95.7
% of CD4+IL-2+ cells
**
*
NS
VZV
NS
VZV
PBMC
Blister

C

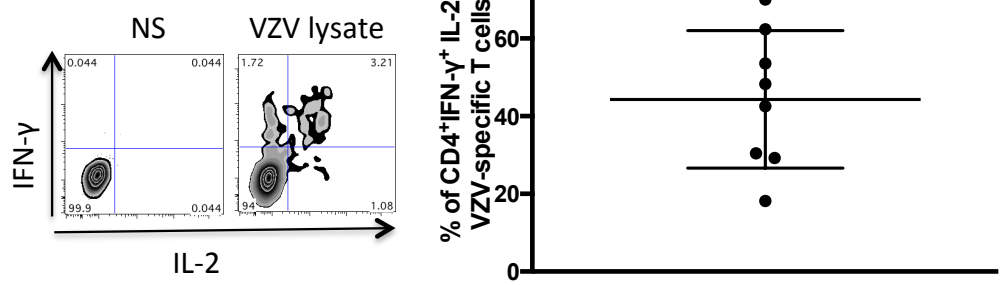

NS
VZV lysate
IFN-γ
IL-2
0.044
0.044
99.9
0.044
1.72
3.21
94
1.08
% of CD4+IFN-γ+ IL-2+ VZV-specific T cells

Figure 5.1 Identification of VZV specific $CD4^+$ T cells at site of cutaneous DTH response by intracellular cytokine staining

Skin suction blisters were raised in young volunteers 7 days after cutaneous VZV antigen injection. PBMCs and blister cells were isolated and re-stimulated for 15 hours with VZV lysate in the presence of Brefeldin A and stained for intracellular expression of IFN-γ (n=15) or IL-2 (n=8). Graphs show cumulative data and the mean value for each group. Each symbol represents one individual. (A) The re-stimulated blister cells contained significantly more $CD4^+IFN\text{-}\gamma^+$ antigen-specific T cells compared to peripheral blood (***p=0.0002, Wilcoxon paired test). There was significantly more $CD4^+IFN\text{-}\gamma^+$ VZV specific cells in the re-stimulated blister and blood samples compared to the non-stimulated controls (*p=0.016 and ***p=0.0001 respectively, Wilcoxon paired test). Representative FACS plots are shown on the left. (B) The re-stimulated blister cells also contained significantly more $CD4^+IL\text{-}2^+$ antigen-specific T cells compared to peripheral blood (**p=0.008, Wilcoxon paired test) and compared to the non-re-stimulated blister fluid sample (*p=0.016, Mann Whitney test). Representative FACS plots are shown on the left. (C) A large proportion of antigen specific T cells secreted both cytokines. Graph shows mean +/- standard deviation (mean 44.3% +/-17.7 S.D). Representative FACS plots are shown on the left.

5.2.2 Assessment of the frequency of VZV specific $CD4^+$ T cells using a MHC class II tetramer

PBMCs and blister cells were isolated 7 days after cutaneous VZV antigen injection in individuals known to be positive for HLA-DRB1*1501. Cells were stained with an HLA-DRB1*1501 restricted IE63 tetramer and analysed by flow cytometry. A higher proportion of $tetramer^+CD4^+$ T cells were identified in the blister cell population compared to peripheral blood (***p=0.0002, Wilcoxon paired test) (Figure 5.2A). Specificity of the tetramer was confirmed by showing a lack of staining with a control tetramer (CLIP) in HLA-DRB1*1501 positive individuals (n=3) and by a lack of HLA-DRB1*1501 restricted IE63 tetramer staining in HLA-DRB1*1501 negative individuals (n=2) (Figure 5.2C,D).

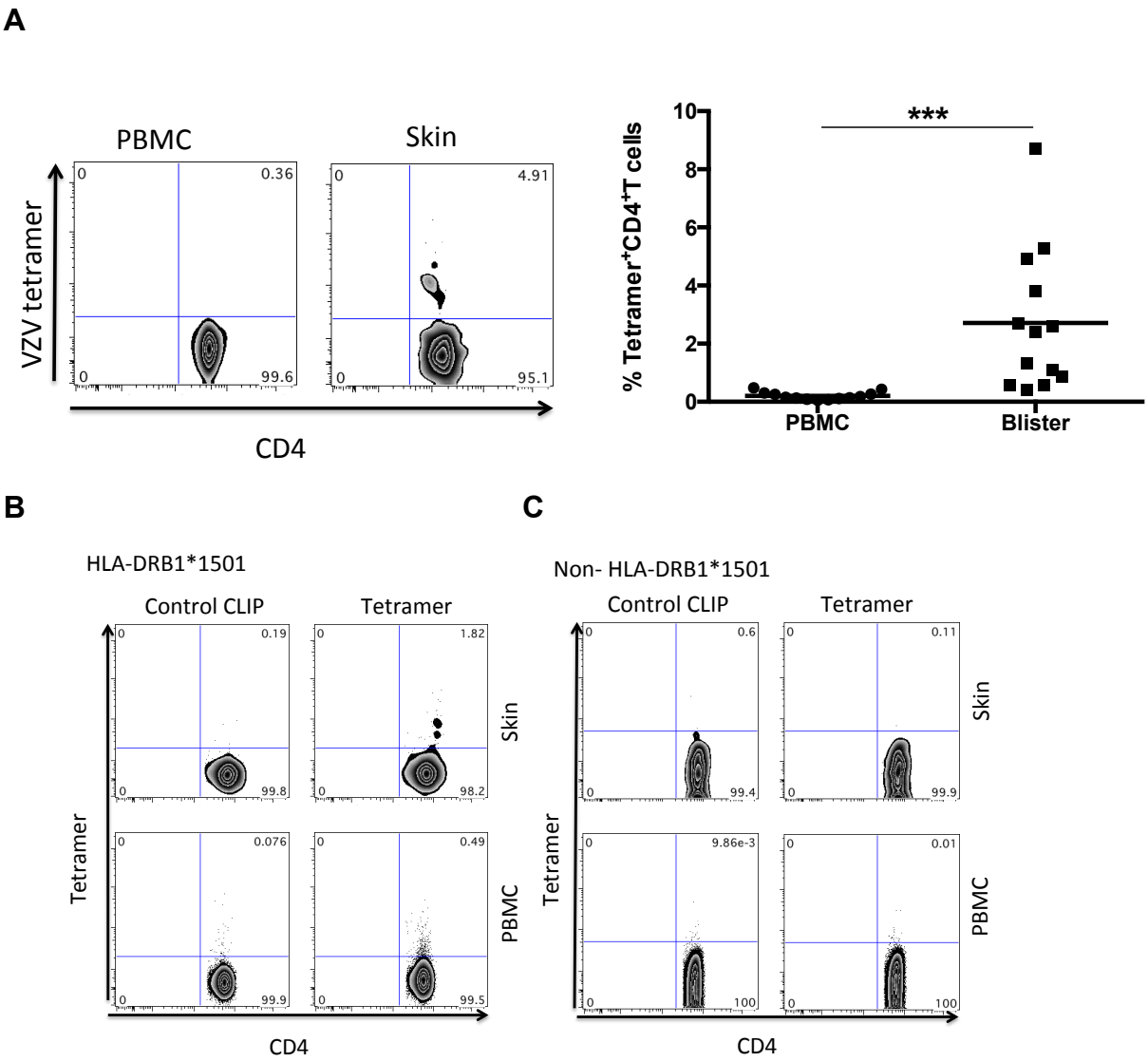


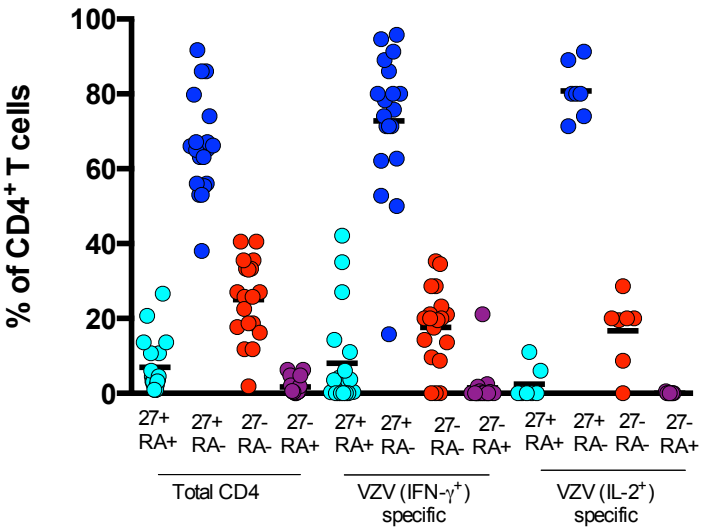
Figure 5.2 Identification of VZV specific $CD4^+$ T cells at site of cutaneous DTH response by class II tetramer

Skin suction blisters were raised in young volunteers (n=13) who were known to be HLA-DRB1*1501 positive 7 days after cutaneous VZV antigen injection. PBMCs and blister cells were isolated and stained with HLA-DRB1*1501 restricted IE63 tetramer. (A) Graph shows cumulative data and the mean value for each group. Each symbol represents one individual. The percentage of $tetramer^+CD4^+$ T cells in the blister fluid was significantly increased compared to peripheral blood (***p=0.0002, Wilcoxon paired test). A representative FACS plot is shown on the left. (B) Skin suction blisters were raised in HLA-DRB1*1501 positive individuals (n=3) 7 days after cutaneous VZV antigen injection. PBMCs and blister cells were isolated and stained with the HLA-DRB1*1501 restricted IE63 tetramer (right panels) and with the control tetramer (CLIP) (left panels). A representative dot plot is shown. (C) Skin suction blisters were raised in HLA-DRB1*1501 negative individuals (n=2). PBMCs and blister cells were isolated and stained as above with the HLA-DRB1*1501 restricted IE63 tetramer (right panels) and the control tetramer (CLIP) (left panel). A representative dot plot is shown.

5.3 Differentiation status of VZV-specific T cells at site of the DTH response

To further characterise the VZV-specific $CD4^+$ T cells in the skin during the DTH response we examined their differentiation status. Blisters cells were retrieved from skin suction blisters 7 days after cutaneous VZV antigen injection in young volunteers. VZV-specific $CD4^+$ T cells were identified by their ability to synthesise IFN-γ or IL-2 after overnight re-stimulation with VZV lysate as described above and cells were stained for the presence of CD27, CD28, and CD45RA. The majority of VZV-specific T cells isolated from the skin were of central memory and effector memory phenotype ($CD45RA^-$ $CD27^+$ and $CD45RA^-CD27^-$ respectively) and were not highly differentiated ($CD27^+CD28^+$) (Figure 5.3).

A



B

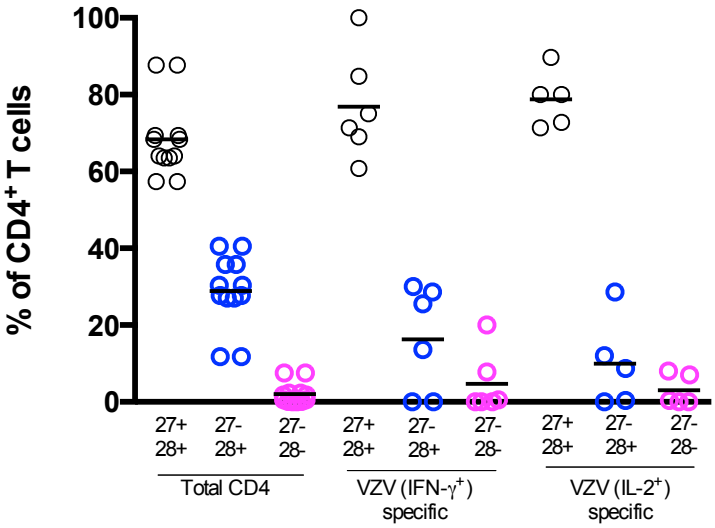


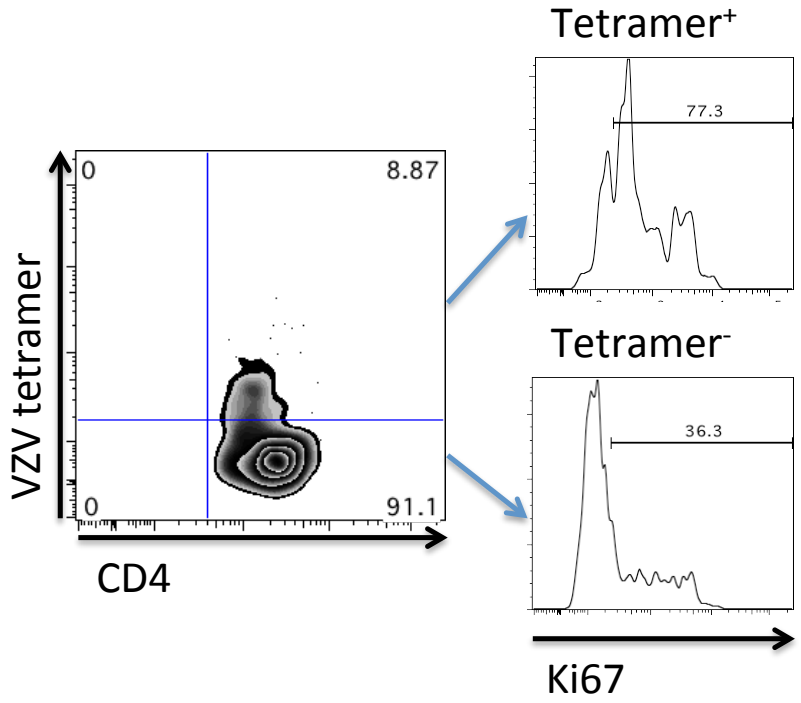
Figure 5.3 Differentiation state of VZV specific $CD4^+$ T cells at site of cutaneous DTH response

Skin suction blisters were induced in young volunteers 7 days after cutaneous VZV antigen injection. Blister cells were isolated and re-stimulated with VZV lysate for 15 hours in the presence of Brefeldin A. Cells were stained for expression of IFN-γ (n=12) and IL-2 (n=7) to identify VZV-specific T cell populations as well as CD27, CD28 and CD45RA, key differentiation markers. Samples were analysed by flow cytometry. (A, B) Graphs show cumulative data. Bars show mean values. Each symbol represents one individual. The majority of cells in both the total $CD4^+$ T cell and $CD4^+$ VZV-specific T cell (either IFN-γ^+ or IL-2^+) populations are of central memory and effector memory phenotype ($CD45RA^-CD27^+$ and $CD45RA^-CD27^-$ respectively) (A) and are not highly differentiated ($CD27^+CD28^+$) (B).

5.4 VZV-specific memory $CD4^+$ T cells proliferate at the site of the DTH response

The accumulation of VZV-specific T cells seen in the skin after cutaneous VZV antigen injection may be the result of recruitment from the circulation and / or local proliferation of recruited or resident VZV-specific T cells in the skin. We have already shown in chapter 4 in skin biopsies taken from young volunteers after VZV skin test that approximately 10-35% of $CD4^+$T cells are proliferating at the site of the cutaneous DTH response by day 7 (Figure 4.5). To confirm that it is VZV-specific $CD4^+$ T cells that are proliferating and contributing to the accumulation of T cells in the skin in response to the VZV skin test, we raised day 7 skin suction blisters after cutaneous VZV antigen injection in young (n=4) volunteers, all of whom were known to be HLA-DRB1*1501 positive. Blister cells were stained with HLA-DRB1*1501 restricted IE63 tetramer and for the expression of Ki67 and analysed by flow cytometry. Proliferation was seen in both tetramer positive and negative $CD4^+$ T cells in the skin. On average approximately 45% of tetramer positive $CD4^+$ T cells were in cell cycle compared to approximately 20% of the tetramer negative $CD4^+$ T cells (*p=0.02, paired t-test) (Figure 5.4). This suggests that local proliferation of VZV-specific $CD4^+$ T cells does occur in the skin. The proliferating tetramer negative T cells would be consistent with activated VZV-specific $CD4^+$ T cells present in the skin after VZV skin test that are responding to VZV peptides other than the immunodominant IE63 peptide. The proliferation rate would be expected to be lower in this tetramer negative population compared to the tetramer positive population due to the presence of non-VZV specific cells.

A



B

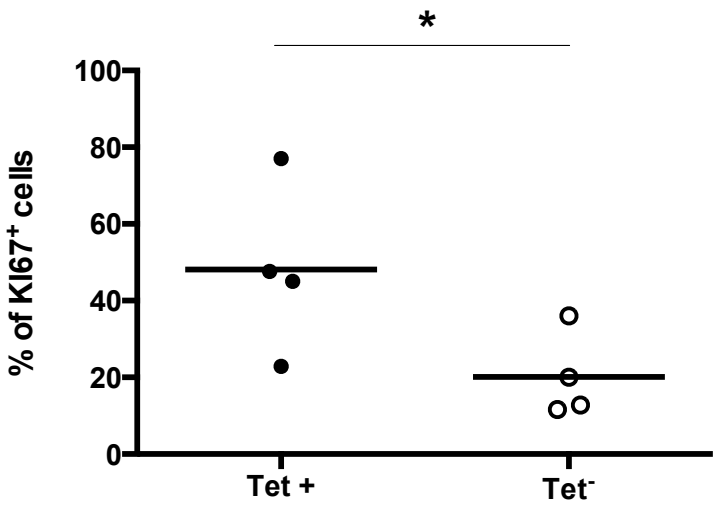


Figure 5.4 VZV specific memory $CD4^+$ T cells proliferate at the site of the cutaneous DTH response

We raised skin suction blisters in young volunteers (n=4), known to be HLA-DRB1*1501 positive, 7 days after cutaneous VZV antigen injection. Blister cells were stained with HLA-DRB1*1501 restricted IE63 tetramer and for the expression of Ki67. Samples were analysed by flow cytometry. (A) The dot plot shows tetramer staining of blister cells. The histograms show Ki67 staining of the tetramer gated populations. (B) Graph shows cumulative data and the mean for each group. Each symbol represents one individual. Tetramer positive $CD4^+$ T cells showed a significantly higher proliferative rate that the tetramer negative $CD4^+$ T cell population (*p=0.02, paired t-test).

5.5 $CD4^+Foxp3^+$ T cells accumulate in the skin after VZV antigen injection and are suggestive of T regulatory cells

We have already shown in chapter 4 in skin biopsies taken from young volunteers after cutaneous VZV antigen challenge that $CD4^+Foxp3^+$ T cells accumulate at the site of the DTH response alongside the memory $CD4^+$ T cell response (Figure 4.7). Activated $CD4^+$ T cells can transiently up-regulate expression of Foxp3, however these cells do not express the typical Treg phenotype CD25hiCD127loCD39hi. Conversely, Tregs do not secrete pro-inflammatory cytokines after activation. Our group has previously shown that $CD4^+Foxp3^+$ T cells isolated from the skin during a DTH response to PPD are consistent with a predominantly T regulatory rather than recently activated T cell population (Vukmanovic-Stejic, Agius et al. 2008). We investigated whether our cutaneous $CD4^+Foxp3^+$ T cells generated during the DTH response to VZV antigen were also in keeping with a T regulatory population.

In order to characterize the phenotype of the $CD4^+Foxp3^+$ T cells that accumulated in the skin after cutaneous VZV skin injection, we raised skin suction blisters in young volunteers 7 days after cutaneous VZV antigen injection. A peripheral blood sample was also taken at the time of blister retrieval. Blister cells and PBMCs were isolated and stained for Foxp3, CD25, CD127 and CD39 expression. Samples were analyzed by flow cytometry. There were significantly more $CD4^+Foxp3^+$ T cells present in the skin at the site of the DTH response than in peripheral blood (****$p<0.0001$, Wilcoxen paired test) (Figure 5.5).

A

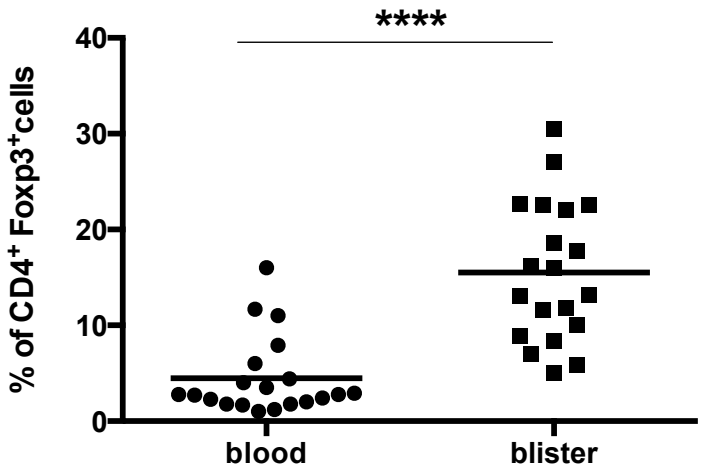


Figure 5.5 $CD4^{+}Foxp3^{+}$ T cells at site of cutaneous DTH response

Skin suction blisters were raised in young volunteers (n=20) 7 days after cutaneous VZV antigen injection. Blister cells and PBMCs were isolated and stained for Foxp3 expression and analysed by flow cytometry. Each symbol represents one individual. Mean is shown for each group. There was a significantly larger proportion of $CD4^{+}Foxp3^{+}$ T cells present in the blister fluid than in peripheral blood (****$p<0.0001$, Wilcoxon paired test).

We found that on average more than 80% of $CD4^{+}Foxp3^{+}$ blister cells expressed high levels of CD25 and high levels of CD39, and that fewer than 10% expressed high levels of CD127; this was significantly different from the $CD4^{+}Foxp3^{-}$ population (****p<0.0001, **p=0.002, ****p<0.0001 respectively, Wilcoxon paired test) (Figure 5.6). Therefore the majority of CD4+Foxp3+ T cells at the site of the DTH response express a typical T regulatory cell phenotype.

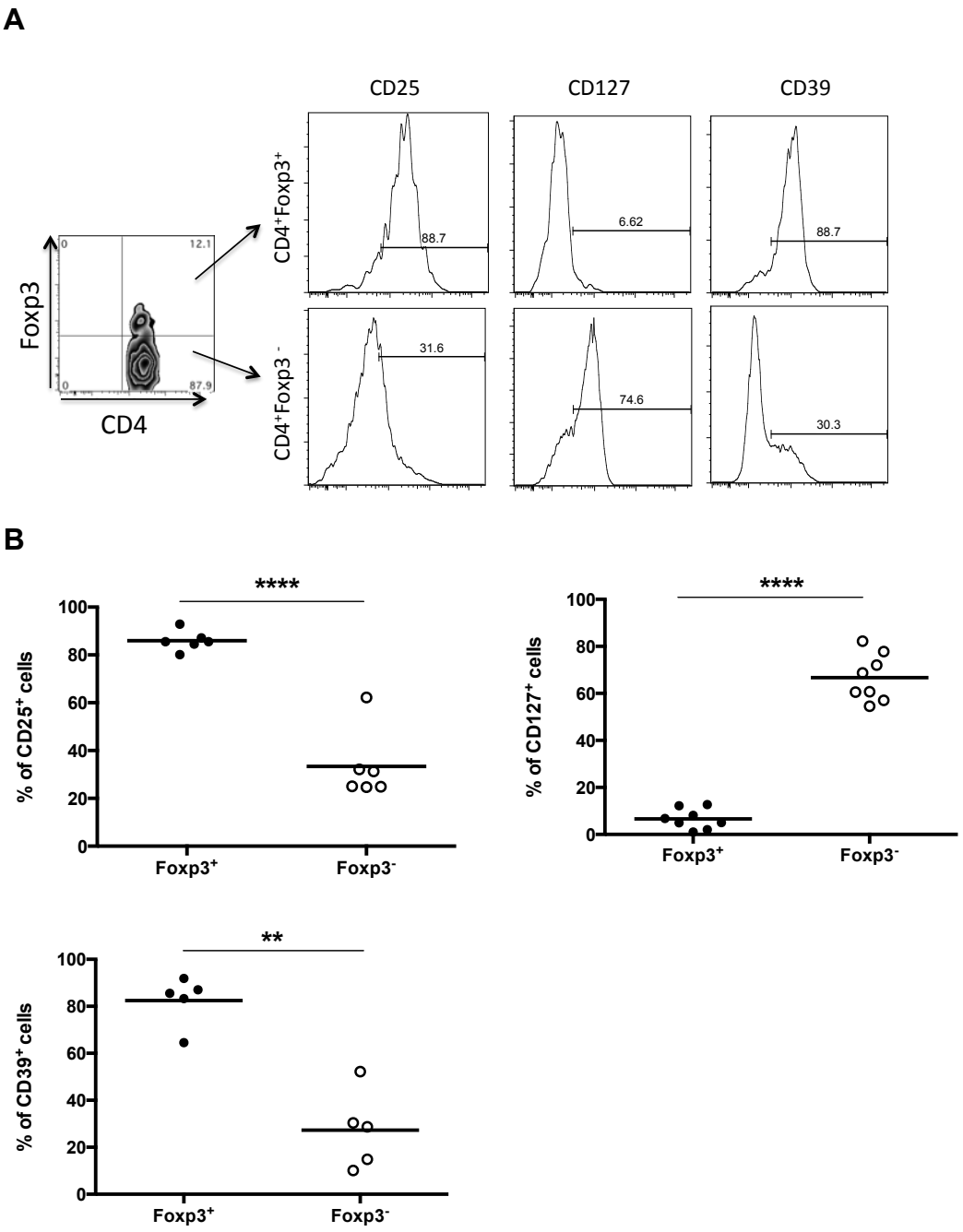


Figure 5.6 Phenotype of $CD4^+Foxp3^+$ T cells at site of cutaneous DTH response

Skin suction blisters were raised in young volunteers 7 days after cutaneous VZV antigen injection. Blister cells were isolated and stained for expression of Foxp3, CD25, CD127 and CD39. Samples were analysed by flow cytometry. (A) The dot plot shows gating strategy to identify $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells (left panel). The histograms show representative expression of CD25, CD39 and CD127 on the gated populations. (B) Graphs show cumulative data and the mean value for each group. Each symbol represents one individual. $CD4^+Foxp3^+$ cells expressed significantly higher levels of CD25 and CD39 and significantly lower levels of CD127 (****$p<0.0001$, **$p=0.002$, ****$p<0.0001$ respectively, Wilcoxon paired test) compared to the $CD4^+Foxp3^-$ population.

One of the hallmarks of T regulatory cells is that they do not secrete pro-inflammatory cytokines after stimulation. To see if this was true of our $CD4^+Foxp3^+$ T cells, we isolated blister cells from skin suction blisters raised in young volunteers 7 days after cutaneous VZV antigen injection and stimulated them *in vitro* with VZV lysate for 15 hours in the presence of Brefeldin A. Cells were stained for intracellular cytokine production (IFN-γ, TNF-α, IL-2 and IL-10) and analyzed by flow cytometry. The $CD4^+Foxp3^+$ T cells produced much less IFN-γ and TNF-α, compared to the $CD4^+$ T cell population and virtually no IL-2. The greatest production of inflammatory cytokine was seen from the $CD4^+Foxp3^-CD127^{lo}$ population that represents the effector memory population (Sauce, Larsen et al. 2007) (Figure 5.7). The lack of inflammatory cytokine production after stimulation suggests that our $CD4^+Foxp3^+$ T cells are functionally consistent with T regulatory cells. Although some T regulatory cells are able to produce IL-10, a suppressive cytokine, we did not see any significant production from our $CD4^+Foxp3^+$ T cell population.

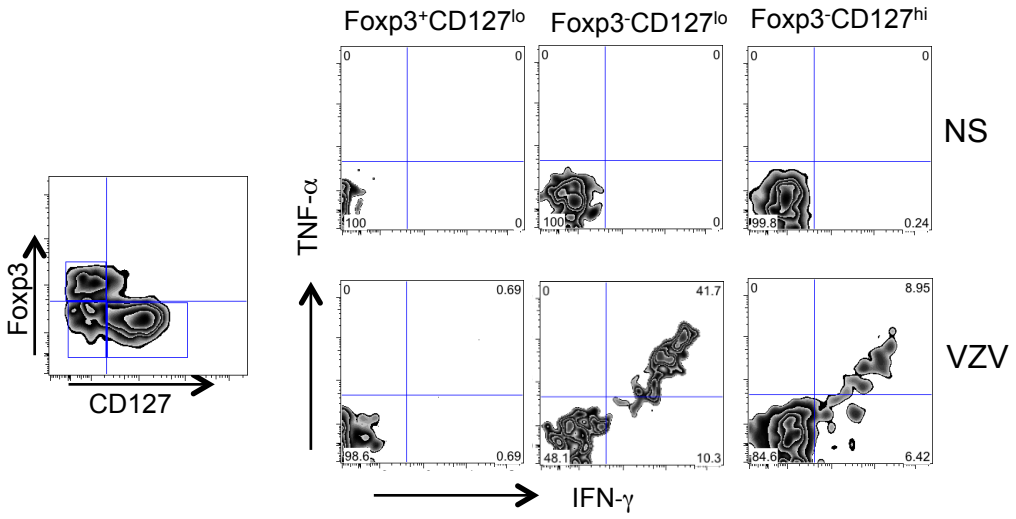


Figure 5.7 Lack of pro-inflammatory cytokine production by $CD4^+Foxp3^+$ T cells at the site of cutaneous DTH response

Skin suction blisters were raised in young volunteers 7 days after cutaneous VZV antigen injection. Blister cells were isolated and stimulated with VZV lysate for 15 hours in the presence of Brefeldin A. Non-stimulated controls were set up in parallel. Cells were stained for expression of Foxp3, CD127, IFN-γ, TNF-α, IL-2 and IL-10. Samples were analyzed by flow cytometry. (A) Dot plot shows gating strategy to identify different Foxp3 and CD127 populations from total $CD4^+$ T cells. Cytokine production is shown in representative dot plots for these subsets.

To determine whether our $CD4^+Foxp3^+$ population exerted a suppressive effect *in vivo* we compared the proportion of $CD4^+$ cells expressing $Foxp3^+$ cells present at the peak of the cellular response (day 7) after VZV injection (Figure 4.7) with the clinical score (Figure 4.1). We found an inverse correlation between the proportion of $CD4^+Foxp3^+$ T cells and the clinical score, indirectly suggesting a suppressive role of the $CD4^+Foxp3^+$T cells population *in vivo* (p=<0.0001, linear regression analysis) (Figure 5.8).

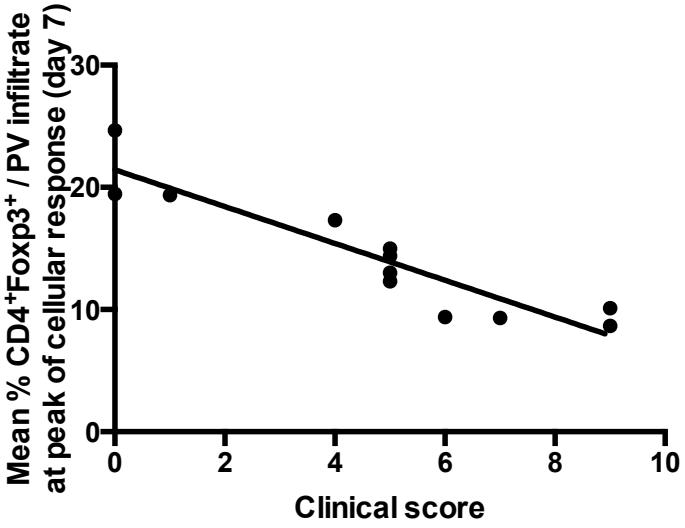


Figure 5.8 Correlation of proportion of Foxp3$^+$ expressing CD4$^+$ cells at the peak of the cellular response to VZV skin test antigen and clinical score

Young and old volunteers (n=12) were injected intra-dermally with 0.02ml of VZV antigen into the medial aspect of the proximal forearm. Clinical score was recorded after 48-72 hours after injection and a 5mm punch biopsy was taken of the site after 7 days. The clinical score is plotted against the mean proportion of Foxp3-expressing CD4$^+$ T cells found in the skin at the peak of the cellular response (day 7) for each individual. There is an inverse correlation between the proportion of CD4$^+$Foxp3$^+$ cells present at the peak of the cellular response and the clinical score (linear regression analysis p=<0.0001).

Therefore the CD4$^+$Foxp3$^+$ population identified in the skin during the DTH response to VZV skin test are phenotypically and functionally in keeping with T regulatory cells. Next we wanted to investigate whether this population displayed specificity for VZV antigen.

5.6 Identification of VZV-specific T regulatory cells in the skin after VZV antigen injection

To determine whether there were VZV-specific T regulatory cells present, we looked to identify cells that positively stained for the HLA-DRB1*1501 restricted IE63 tetramer in the CD4$^+$Foxp3$^+$ population. To do this we raised skin suction blisters 7 days after cutaneous VZV antigen injection in young

volunteers known to be positive for HLA-DRB1*1501. Tetramer positive cells could be identified in the $CD4^+Foxp3^+$ T cell population. In most cases, 0-5% of cells, in both the $CD4^+$ and $CD4^+Foxp3^+$ populations, were tetramer positive i.e. VZV-specific (Figure 5.9). This similarity in proportion may hint at the populations arising in parallel during the DTH response as was seen in skin biopsies taken from young volunteers after VZV skin test (Figure 4.7).

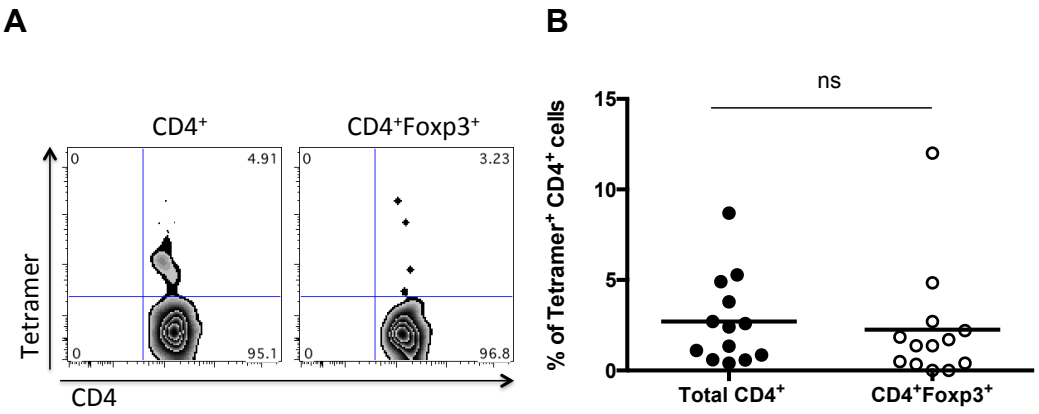


Figure 5.9 Identification of VZV specific (tetramer+) T regulatory cells at site of cutaneous DTH response

Skin suction blisters were raised 7 days after cutaneous VZV antigen injection in young volunteers (n=13) known to be HLA-DRB1*1501 positive. Blister cells were isolated and stained with HLA-DRB1*1501 restricted IE63 tetramer and for Foxp3 expression. Samples were analyzed by flow cytometry. (A) Representative dot plot shows gating strategy to identify tetramer positive cells in both total $CD4^+$ and $CD4^+Foxp3^+$ T cell populations. (B) Graph shows cumulative data and the mean value for each population. Each symbol represents one individual. The percentage of tetramer positive T cells is similar in both the total $CD4^+$ and $CD4^+Foxp3^+$ T cell populations (p=0.6 Wilcoxon paired test).

To characterise the phenotype of these $CD4^+Foxp3^+tetramer^+$ T cells we compared the expression of CD25, CD127 and CD39 on the $tetramer^+Foxp3^+$ population with the $tetramer^+Foxp3^-$ and the $tetramer^-Foxp3^{+/-}$ populations (Figure 5.10A). Within the $Foxp3^+$ populations the majority of cells, both positive and negative for tetramer expressed high levels of CD25 and CD39 typical of T regulatory cells or an activated state. This was also true of the $tetramer^+Foxp3^-$Tcells, consistent with memory VZV-specific cells that we would expect to be activated. This is in contrast to the $tetramer^-Foxp3^-$T cells

that may be neither activated, due to a lack of VZV specificity, nor T regulatory in nature. Within the $Foxp3^+$ population, more tetramer negative cells had a low expression of CD127 than did the tetramer positive population, suggesting a predominantly T regulatory phenotype in the former population but a combination of T regulatory and activated cells in the latter. Some of the activated $tetramer^+Foxp3^+$ cells may be in a state of transition from a VZV-specific effector T cell to a VZV-specific regulatory T cells.

Looking at the combined expression of CD25 and CD127 in Foxp3 expressing $CD4^+$ cells in both the tetramer + and - populations, we found that the majority of $Foxp3^+$ cells within the tetramer negative population are $CD25^{hi}CD127^{lo}$ rather than $CD25^{hi}CD127^{hi}$, suggestive of a T regulatory phenotype. In the tetramer positive population, there is again a predominance of $CD25^{hi}CD127^{lo}$ cells (~60%) consistent with the presence of a VZV-specific T regulatory cells but also a relevant proportion of $CD25^{hi}CD127^{hi}$ cells again pointing to a population of either activated T cells or those that may be in transition to an iTreg phenotype (Figure 5.7 D).

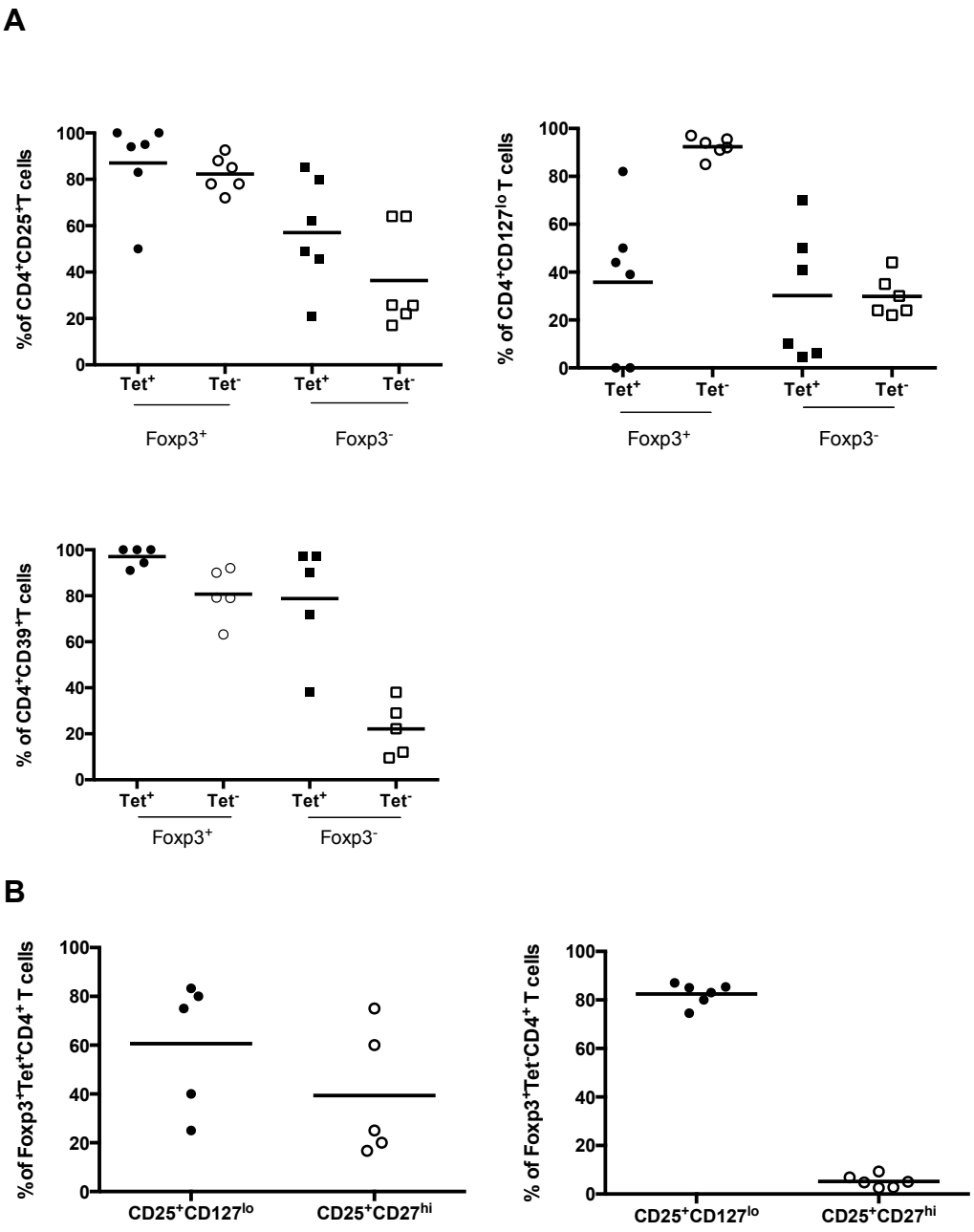


Figure 5.10 Phenotype of $CD4^+Foxp3^+$tetramer$^+$ T cells at site of cutaneous DTH response

Tetramer positive and negative, $Foxp3^+$ and $Foxp3^-$, $CD4^+$ T cell populations were further analyzed for their expression of CD25, CD127 and CD39 (n=5-6). Graphs show cumulative data and the mean value for each subset. Each symbol represents one individual. (A) The majority of $Foxp3^+$ T cells in both the tetramer positive and negative populations express CD25 and CD39. The majority of $Foxp3^+$ tetramer negative T cells also are $CD127^{lo}$ whilst there is a more mixed population in the $Foxp3^+$ tetramer positive population. (B) The majority of $CD4^+Foxp3^+$tetramer$^-$ T cells are $CD25^{hi}CD127^{lo}$ in keeping with a Treg phenotype (right panel). The majority of $CD4^+Foxp3^+$tetramer$^+$ cells are also $CD25^{hi}CD127^{lo}$ (left panel). However, a relevant population that is $CD25^+CD127^{hi}$ is also present.

5.7 Discussion

We used a skin suction blister technique to allow further characterisation of the cellular response at the site of VZV antigen injection shown in chapter 4. We found that approximately 5% of $CD4^+$ T cells in the skin after VZV antigen injection were VZV specific identified by cytokine secretion compared to approximately 3% identified by class II tetramer. However, it is difficult to accurately determine the exact proportion of VZV specific cells at the site of the DTH response. The proportion of cytokine producing T cells is likely to be an underestimate as some of the specific cells recovered from the injected skin site may be refractory to further activation *in vitro* while others may not have the capacity to secrete IL-2 or IFN-γ or be inhibited from doing so by Tregs. Detection by tetramer is also likely to underestimate the true number as it will not detect VZV specific $CD4^+$ T cells of different specificities that are also present within the skin after challenge. Despite this, it can be seen that a significant proportion of the infiltrating cells are antigen specific.

Production of IL-2 and IFN-γ by VZV specific T cells is in keeping with a combination of central memory and effector cells, confirmed by studying surface markers of differentiation. However it should be noted that in both VZV-specific populations regardless of whether they secrete IFN-γ or IL-2, the predominant population is consistent with a central memory phenotype. As within peripheral blood, the vast majority of VZV-specific cells are not highly differentiated ($CD27^+CD28^+$) although there is a slightly higher proportion of more differentiated cells ($CD27^-CD28^+$) in the skin (7.7% vs 16.3% IFN-γ^+ and 7.0% vs 9.9% IL-2^+ of blood and blister cells respectively). This is perhaps in keeping with the larger effector memory population seen in the skin compared to the blood (approximately 20% vs. 10% respectively) and may reflect more frequent involvement of this population in controlling clinically silent reactivations of VZV as it travels down peripheral nerves into the skin. Similarly herpes simplex virus 1 (HSV-1) specific T cells isolated from trigeminal ganglia are also more highly differentiated than those in peripheral blood (Verjans, Hintzen et al. 2007). Differentiation was studied on cytokine producing VZV-specific T cells rather than those isolated by class II tetramer

as the former was a larger population and so more suitable for analysis of subpopulations.

Using our blister technique to isolate leukocytes from the site of VZV antigen challenge in combination with a class II tetramer we were able to confirm that there is proliferation of VZV-specific cells in the skin, as suggested by histology (Figure 4.5). Approximately 45% of tetramer positive cells were proliferating on day 7, as well as 20% of tetramer negative T cells. This latter is suggestive of proliferation of $CD4^+$ T cells responding to other VZV peptides. Therefore while some antigen-specific T cells are recruited from the circulation, proliferation of VZV-specific T cells at the site of VZV challenge also contributes to the accumulation of VZV-specific cells. Among these accumulating cells are some $CD4^+Foxp3^+$ cells. By histology we identified that approximately 10% of the total number of $CD4^+$ T cells/ PV infiltrate were $Foxp3^+$ at day 7 after VZV injection although by our suction blister technique a mean of 16% were $Foxp3^+$. This difference may reflect a higher proportion of $Foxp3^+$ T cells outside the immediate PV infiltrates examined by histology, e.g. around hair follicles (Clark and Kupper 2007) and provide a more accurate representation of the total skin area involved in the response. The proportion of $CD4^+Foxp3^+$ T cells in the skin after VZV injection was significantly higher than in peripheral blood from the same individual. This reflects the local accumulation of these cells in part due to local proliferation as shown by histology (Figure 4.8) as well as new transient expression of Foxp3 in activated T cells and possibly generation of iTregs from the effector T cells.

We were able to show that $CD4^+Foxp3^+$ T cells in the skin isolated from suction blister typically expressed high levels of CD25, CD39 and low levels of CD127, in keeping with a Treg phenotype. $CD4^+Foxp3^+$ T cells produced negligible IL-2, IFN-γ and TNF-α compared to $CD4^+Foxp3^-$ T cells. Nor did they secrete IL-10, an immune suppressive cytokine typically produced by Tregs in the intestinal mucosa (Maynard, Harrington et al. 2007; Rubtsov, Rasmussen et al. 2008). The immune suppressive nature of the $CD4^+Foxp3^+$

cells is indirectly conferred by the inverse correlation between the proportion of these cells and the clinical response to VZV antigen. Therefore in keeping with previous work studying the response to the Mantoux test, $CD4^+Foxp3^+$ T cells that accumulate at the site of a DTH response to VZV are phenotypically and functionally suggestive of Tregs (Vukmanovic-Stejic, Agius et al. 2008) and most likely function to regulate the effector T cell response. Using the class II tetramer we were also able to show that approximately 3% of both the total $CD4^+$ and $CD4^+Foxp3^+$ populations were specific for VZV supporting the hypothesis that they accumulate in parallel. Phenotype analysis of the tetramer specific $CD4^+Foxp3^+$ T cells showed that the majority were in keeping with Tregs although some would be compatible with either activated $CD4^+$ T cells which transiently express Foxp3 and high levels of both CD25 and CD127 as well as cells which may be in a state of transition from a T effector to a Treg cell with a varying level of CD127 expression.

Generation of Tregs from memory T cells during an immune response has been proposed as a mechanism for ensuring adequate regulation of the immune response (Akbar, Vukmanovic-Stejic et al. 2007). It also provides an explanation for how Tregs may be maintained over the lifetime of an individual given that the number of naïve nTregs emigrating from the thymus declines with age (Booth, McQuaid et al. 2010) and that Tregs are unlikely to be a long-lived population. Tregs are a highly proliferative population but probably with limited replicative capacity due to extensive telomere erosion and poor induction of telomerase compared to total CD4 T cells (Vukmanovic-Stejic, Zhang et al. 2006). Furthermore it has been shown that Tregs express lower levels of the antiapoptotic molecule Bcl-2 rendering them susceptible to apoptosis (Vukmanovic-Stejic, Zhang et al. 2006). Microarray analysis of RNA has also shown that gene expression profiles seen in $CD45RO^+$ Tregs (i.e. activated Tregs) more closely resemble those of naïve and memory T conventional cells than naïve nTregs, indicating that from a genomic perspective, converted conventional T cells may make up a large proportion of the memory Treg pool (Booth, McQuaid et al. 2010). Our data above shows that VZV-specific Tregs and effector T cells share antigen specificity

and arise in parallel at the site of antigen challenge supporting the hypothesis that iTregs may be derived from memory effector cells during an immune response. If this were the case it is feasible that clinically silent reactivations of VZV in dorsal root ganglia migrating down peripheral nerves would generate new population of Tregs with each episode which may alter the balance of these two populations in favour of Tregs such that they are able to suppress the effector response allowing a clinical episode of herpes zoster to occur. Alternatively Tregs and naïve T cells may be generated with the same antigen specificity for non-self proteins within the thymus and become activated in tandem during an immune response.

Chapter 6: Summary and future directions

The aim of this study was to characterise the effects of ageing on the immune system in the skin in healthy old individuals. To do this we used the delayed type hypersensitivity response as a model for a memory T cell response. Our antigen of interest was VZV. Practically all adults in the UK acquire immunity to VZV through infection in childhood when it causes chickenpox. VZV-CMI declines with increasing age and is associated with an increased risk of VZV reactivation. VZV reactivation may clinically present with shingles.

To recruit elderly volunteers into our study we used a modified version of the SENIEUR protocol (Ligthart, Corberand et al. 1984) with a view to exclude significant co-morbidity and pharmacological interference but select a representative rather than exceptional group of old individuals. Study of a similarly 'almost healthy' elderly population has shown significant differences in immune function compared to young individuals, not seen in the healthy old (Mysliwska, Bryl et al. 1999). We performed both skin biopsies and suction blisters to study the immune response to VZV antigen. Histological analysis of skin sections allowed us to identify the nature and kinetics of the cellular infiltrate while cells isolated from suction blisters allowed us to determine T cell specificity, function and phenotype.

We show that elderly individuals injected with VZV antigen have a weaker clinical response assessed by erythema, palpability and diameter of induration. This is despite similar numbers of circulating VZV specific $CD4^+$ T cells. There are however fewer circulating IFN-γ^+CD4^+ T in the elderly. The cellular DTH response in the skin is defective in elderly individuals demonstrated by a lack of T cell accumulation at the site of antigen challenge.

It is known that in response to trauma-induced DAMPs and pathogen induced PAMPs there is activation of PRRs as well as other receptors on cells of the innate immune system (Matzinger 2002; Medzhitov and Janeway 2002). Downstream signalling results in the production of pro-inflammatory cytokines

and chemokines, which are important for initiating an inflammatory response (Blackwell and Christman 1997). One element of this is activation of dermal endothelium to express adhesion molecules required for the recruitment of circulating memory T cells and other leukocytes. Recent evidence suggests that, like innate immune cells, tissue resident memory T cells may also perform an 'alarm function' after antigen recognition and through the secretion of pro-inflammatory cytokines, induce the expression of chemokines to recruit circulating memory T cells (Strutt, McKinstry et al. 2010; Schenkel, Fraser et al. 2013). The early recruitment of T cells from the circulation is not antigen specific (Ghani, Feuerer et al. 2009) but is vital for subsequent T cell accumulation (Hwang, Yamanouchi et al. 2004). It is thought that this initial wave of recruitment will include some antigen specific T cells, which undergo activation and proliferation in the skin. These activated antigen-specific cells would then provide an additional source of pro-inflammatory cytokines such as TNF-α and IFN-γ to further enhance endothelial activation such that there is larger scale recruitment of memory T cells. Recruited DCs would provide a larger pool of APCs to present antigen to the increasing numbers of antigen specific T cells (McLachlan, Catron et al. 2009). Tregs accumulate alongside effector T cells and provide a means of regulating the response (Vukmanovic-Stejic, Agius et al. 2008; McLachlan, Catron et al. 2009)

We propose that there is a population of VZV-specific T cells resident in normal skin and that these are involved in the generation of an antigen specific memory response. There may be fewer functioning VZV specific T cells in the skin of elderly individuals compared to young individuals, alongside an increased proportion of Tregs that results in an impaired memory T cell response. We suggest that the innate immune response is intact demonstrated by similar expression of E-selectin on dermal endothelium and recruitment of neutrophils in young and old individuals 6 hours after injection of either VZV antigen or normal saline. Evidence for an early antigen specific defect is seen at 6 hours when there is an increase in the number of $CD11c^+$ DCs only in young individuals and only after VZV antigen injection. We hypothesise that with fewer VZV antigen specific cells

available to or able to undergo activation in the skin of the elderly there is lesser production of chemokines to attract circulating DCs and pro-inflammatory cytokines such as IFN-γ to enhance endothelial activation. The failure to increase E-selectin expression beyond 6 hours in the old individuals would be in keeping with this. A cycle is therefore set up whereby VZV specific T cells in the skin do not adequately enhance activation of dermal endothelium nor produce chemokines needed to recruit DCs and T cells from the circulation to amplify the memory response. Recruitment of macrophages may be similarly impaired.

The number of functioning VZV specific T cells in the elderly may be reduced as a consequence of functional exhaustion in response to repeated subclinical reactivation of VZV along peripheral nerves. Alternatively VZV specific cells in the skin of the elderly may come under increased suppression from the higher proportion of Tregs present. In support of this hypothesis we showed that a small subset of elderly people who have clinical responses resembling those seen in the young, also have similar proportions of $CD4^{+}Foxp3^{+}$ T cells in normal skin. Although we identified Tregs that shared antigen specificity with the effector T cell population in the young during the DTH response, it is more probable that the Tregs in normal skin act in a non-specific manner. These Tregs are perfectly placed to interfere with the earliest elements of T cell activation such as antigen presentation by dendritic cells. Tregs are also able to inhibit macrophage function including TNF-α secretion (Taams, van Amelsfort et al. 2005; Tiemessen, Jagger et al. 2007). We propose that it is the balance of Tregs versus Teffector cells that is important in determining the outcome of the DTH response (Lages, Suffia et al. 2008). This balance could be altered by the shingles vaccine to increase either the pool of VZV specific T cells in the skin or those available for recruitment from the circulation with a subsequent increase in the cutaneous response to VZV antigen in the elderly (Takahashi, Kamiya et al. 2001). It is not known why Tregs accumulate in the skin of old people but it may in part reflect a UV induced Treg population (Schwarz 2008) that has accumulated over years. UV induced Tregs are able to alter the function of dendritic cells through secretion of IL-10 such that they

become ‘tolerogenic’ and induce the formation of further Tregs (Schwarz, Navid et al. 2011).

In addition or as an alternative to the possibility of increased suppression of innate cell function by Tregs in the elderly, activation of VZV specific T cells in the skin may also be impaired secondary to age-related defects of innate cells involved in the response. Defects in TLR function with ageing have been reported in both macrophages and dendritic cells (Shaw 2011)(van Duin, Allore et al. 2007; Agius, Lacy et al. 2009) and may contribute to a reduced level of early pro-inflammatory cytokine production in the elderly. Age-related defects in macrophage polarization with suppression of both M1 (pro-inflammatory) and M2 (anti-inflammatory) responses have also been reported (Mahbub, Deburghgraeve et al. 2012).

An alternative hypothesis to the existence of a skin resident population of VZV specific cells that initiates the specific response is that antigen-specific T cells from the circulation are recruited non-specifically to the site of inflammation and then initiate the antigen specific response after encountering antigen. It has been proposed by other work (Ghani, Feuerer et al. 2009) that early danger signals trigger low-level, non-antigen specific infiltration of circulating T cells and other leucocytes. Local antigen presented by resident or recruited APCs would initiate the activation of a few antigen-specific T cells within the infiltrate and act as ‘pioneering cells’ (Ghani, Feuerer et al. 2009). These activated antigen-specific T cells could then produce pro-inflammatory mediators such as IFN-γ and TNF-α either directly or indirectly via macrophages to activate endothelium and stimulate chemokine-producing cells to promote recruitment of large numbers of T cells and other leukocytes. Antigen-specific T cells could undergo proliferation in the skin after activation. Under these circumstances, since there are fewer functioning VZV-specific cells in the circulation to be recruited into the skin in old individuals, there would be a diminished accumulation of T cells, as is shown in our results. These two hypotheses mentioned above are not mutually exclusive and we feel that it is likely that the reduced number of circulating VZV specific

$CD4^+IFN\gamma^+$ cells in the elderly compounds an earlier defect in the initial activation of VZV specific T cells in the skin resulting in reduced T cell accumulation.

To investigate these hypotheses further it would be of interest to determine the number of VZV specific T cells isolated from normal skin in the old and young and their phenotype to identify a T_{RM} resident-like population. It would be important to assess the ability of these cells to secrete pro-inflammatory cytokines after *in vitro* stimulation with VZV lysate both in the presence and absence of the Tregs to exclude the possibility that VZV specific T cells present in normal skin may be intrinsically defective in the elderly. Production of cytokines, chemokines and activation of pro-inflammatory or anti-inflammatory pathways could be investigated by transcriptional analysis of the skin after VZV antigen injection.

References

Abendroth, A., I. Lin, et al. (2001). "Varicella-zoster virus retains major histocompatibility complex class I proteins in the Golgi compartment of infected cells." J Virol **75**(10): 4878-4888.

Aderem, A. and D. M. Underhill (1999). "Mechanisms of phagocytosis in macrophages." Annu Rev Immunol **17**: 593-623.

Agace, W. (2010). "Generation of gut-homing T cells and their localization to the small intestinal mucosa." Immunol Lett **128**(1): 21-23.

Agius, E., K. E. Lacy, et al. (2009). "Decreased TNF-alpha synthesis by macrophages restricts cutaneous immunosurveillance by memory CD4+ T cells during aging." J Exp Med **206**(9): 1929-1940.

Ahmed, A. R. and D. A. Blose (1983). "Delayed-type hypersensitivity skin testing. A review." Arch Dermatol **119**(11): 934-945.

Ahmed, R. and D. Gray (1996). "Immunological memory and protective immunity: understanding their relation." Science **272**(5258): 54-60.

Akbar, A. N. and J. M. Fletcher (2005). "Memory T cell homeostasis and senescence during aging." Curr Opin Immunol **17**(5): 480-485.

Akbar, A. N., J. R. Reed, et al. (2013). "Investigation of the cutaneous response to recall antigen in humans in vivo." Clin Exp Immunol **173**(2): 163-172.

Akbar, A. N., L. Terry, et al. (1988). "Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells." J Immunol **140**(7): 2171-2178.

Akbar, A. N. and M. Vukmanovic-Stejic (2007). "Telomerase in T lymphocytes: use it and lose it?" J Immunol **178**(11): 6689-6694.

Akbar, A. N., M. Vukmanovic-Stejic, et al. (2007). "The dynamic co-evolution of memory and regulatory CD4+ T cells in the periphery." Nat Rev Immunol **7**(3): 231-237.

Akimova, T., U. H. Beier, et al. (2011). "Helios expression is a marker of T cell activation and proliferation." PLoS One **6**(8): e24226.

Allan, S. E., S. Q. Crome, et al. (2007). "Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production." Int Immunol **19**(4): 345-354.

Angel, C. E., E. George, et al. (2006). "Cutting edge: CD1a+ antigen-presenting cells in human dermis respond rapidly to CCR7 ligands." J Immunol **176**(10): 5730-5734.
Angel, C. E., A. Lala, et al. (2007). "CD14+ antigen-presenting cells in human dermis are less mature than their CD1a+ counterparts." Int Immunol **19**(11): 1271-1279.
Asano, M. S. and R. Ahmed (1996). "CD8 T cell memory in B cell-deficient mice." J Exp Med **183**(5): 2165-2174.
Asanuma, H., M. Sharp, et al. (2000). "Frequencies of memory T cells specific for varicella-zoster virus, herpes simplex virus, and cytomegalovirus by intracellular detection of cytokine expression." J Infect Dis **181**(3): 859-866.
Aspinall, R. and D. Andrew (2000). "Thymic involution in aging." J Clin Immunol **20**(4): 250-256.
Auffray, C., M. H. Sieweke, et al. (2009). "Blood monocytes: development, heterogeneity, and relationship with dendritic cells." Annu Rev Immunol **27**: 669-692.
Autissier, P., C. Soulas, et al. (2010). "Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans." Cytometry A **77**(5): 410-419.
Azukizawa, H., A. Dohler, et al. (2011). "Steady state migratory RelB+ langerin+ dermal dendritic cells mediate peripheral induction of antigen-specific CD4+ CD25+ Foxp3+ regulatory T cells." Eur J Immunol **41**(5): 1420-1434.
Azuma, M., J. H. Phillips, et al. (1993). "CD28- T lymphocytes. Antigenic and functional properties." J Immunol **150**(4): 1147-1159.
Baba, K., H. Yabuuchi, et al. (1978). "Studies with live varicella vaccine and inactivated skin test antigen: protective effect of the vaccine and clinical application of the skin test." Pediatrics **61**(4): 550-555.
Bacchetta, R., L. Passerini, et al. (2006). "Defective regulatory and effector T cell functions in patients with FOXP3 mutations." J Clin Invest **116**(6): 1713-1722.

Bajenoff, M., O. Wurtz, et al. (2002). "Repeated antigen exposure is necessary for the differentiation, but not the initial proliferation, of naive CD4(+) T cells." J Immunol **168**(4): 1723-1729.
Banchereau, J. and R. M. Steinman (1998). "Dendritic cells and the control of immunity." Nature **392**(6673): 245-252.
Baron, V., C. Bouneaud, et al. (2003). "The repertoires of circulating human CD8(+) central and effector memory T cell subsets are largely distinct." Immunity **18**(2): 193-204.
Basham, T. Y. and T. C. Merigan (1983). "Recombinant interferon-gamma increases HLA-DR synthesis and expression." J Immunol **130**(4): 1492-1494.
Basran, A., M. Jabeen, et al. (2013). "Roles of neutrophils in the regulation of the extent of human inflammation through delivery of IL-1 and clearance of chemokines." J Leukoc Biol **93**(1): 7-19.
Bennett, C. L., J. Christie, et al. (2001). "The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3." Nat Genet **27**(1): 20-21.
Berard, M. and D. F. Tough (2002). "Qualitative differences between naive and memory T cells." Immunology **106**(2): 127-138.
Berg, E. L., T. Yoshino, et al. (1991). "The cutaneous lymphocyte antigen is a skin lymphocyte homing receptor for the vascular lectin endothelial cell-leukocyte adhesion molecule 1." J Exp Med **174**(6): 1461-1466.
Bertini, R., M. Sironi, et al. (1992). "Inhibitory effect of recombinant intracellular interleukin 1 receptor antagonist on endothelial cell activation." Cytokine **4**(1): 44-47.
Bevan, M. J. (2011). "Memory T cells as an occupying force." Eur J Immunol **41**(5): 1192-1195.
Bevilacqua, M. P. (1993). "Endothelial-leukocyte adhesion molecules." Annu Rev Immunol **11**: 767-804.
Bevilacqua, M. P., J. S. Pober, et al. (1987). "Identification of an inducible endothelial-leukocyte adhesion molecule." Proc Natl Acad Sci U S A **84**(24): 9238-9242.
Bilate, A. M. and J. J. Lafaille (2012). "Induced CD4+Foxp3+ regulatory T cells in immune tolerance." Annu Rev Immunol **30**: 733-758.

Black, A. P., M. R. Ardern-Jones, et al. (2007). "Human keratinocyte induction of rapid effector function in antigen-specific memory CD4+ and CD8+ T cells." Eur J Immunol **37**(6): 1485-1493.
Blackburn, E. H. (2005). "Telomeres and telomerase: their mechanisms of action and the effects of altering their functions." FEBS Lett **579**(4): 859-862.
Blackwell, T. S. and J. W. Christman (1997). "The role of nuclear factor-kappa B in cytokine gene regulation." Am J Respir Cell Mol Biol **17**(1): 3-9.
Blanchet, X., M. Langer, et al. (2012). "Touch of chemokines." Front Immunol **3**: 175.
Blattman, J. N., R. Antia, et al. (2002). "Estimating the precursor frequency of naive antigen-specific CD8 T cells." J Exp Med **195**(5): 657-664.
Boissonnas, A., A. Scholer-Dahirel, et al. (2010). "Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes." Immunity **32**(2): 266-278.
Bonanni, P., J. Breuer, et al. (2009). "Varicella vaccination in Europe - taking the practical approach." BMC Med **7**: 26.
Booth, N. J., A. J. McQuaid, et al. (2010). "Different proliferative potential and migratory characteristics of human CD4+ regulatory T cells that express either CD45RA or CD45RO." J Immunol **184**(8): 4317-4326.
Borsellino, G., M. Kleinewietfeld, et al. (2007). "Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression." Blood **110**(4): 1225-1232.
Boughton, B. and W. G. Spector (1963). "Histology of the tuberculin reaction in guinea-pigs." J Pathol Bacteriol **85**: 371-381.
Boyman, O., H. P. Hefti, et al. (2004). "Spontaneous development of psoriasis in a new animal model shows an essential role for resident T cells and tumor necrosis factor-alpha." J Exp Med **199**(5): 731-736.
Bromley, S. K., S. Y. Thomas, et al. (2005). "Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics." Nat Immunol **6**(9): 895-901.
Bruder, D., M. Probst-Kepper, et al. (2004). "Neuropilin-1: a surface marker of regulatory T cells." Eur J Immunol **34**(3): 623-630.

Brunkow, M. E., E. W. Jeffery, et al. (2001). "Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse." Nat Genet **27**(1): 68-73.
Burke, B. L., R. W. Steele, et al. (1982). "Immune responses to varicella-zoster in the aged." Arch Intern Med **142**(2): 291-293.
Busse, D., M. de la Rosa, et al. (2010). "Competing feedback loops shape IL-2 signaling between helper and regulatory T lymphocytes in cellular microenvironments." Proc Natl Acad Sci U S A **107**(7): 3058-3063.
Campbell, D. J. and E. C. Butcher (2002). "Rapid acquisition of tissue-specific homing phenotypes by CD4(+) T cells activated in cutaneous or mucosal lymphoid tissues." J Exp Med **195**(1): 135-141.
Campbell, J. J., G. Haraldsen, et al. (1999). "The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells." Nature **400**(6746): 776-780.
Campbell, J. J., J. Hedrick, et al. (1998). "Chemokines and the arrest of lymphocytes rolling under flow conditions." Science **279**(5349): 381-384.
Campbell, J. J., K. E. Murphy, et al. (2001). "CCR7 expression and memory T cell diversity in humans." J Immunol **166**(2): 877-884.
Campbell, J. J., D. J. O'Connell, et al. (2007). "Cutting Edge: Chemokine receptor CCR4 is necessary for antigen-driven cutaneous accumulation of CD4 T cells under physiological conditions." J Immunol **178**(6): 3358-3362.
Cao, X., S. F. Cai, et al. (2007). "Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance." Immunity **27**(4): 635-646.
Castle, S. C., K. Uyemura, et al. (2001). "The SENIEUR Protocol after 16 years: a need for a paradigm shift?" Mech Ageing Dev **122**(2): 127-130.
Cepek, K. L., S. K. Shaw, et al. (1994). "Adhesion between epithelial cells and T lymphocytes mediated by E-cadherin and the alpha E beta 7 integrin." Nature **372**(6502): 190-193.
Cevenini, E., D. Monti, et al. (2013). "Inflamm-ageing." Curr Opin Clin Nutr Metab Care **16**(1): 14-20.

Chidrawar, S., N. Khan, et al. (2009). "Cytomegalovirus-seropositivity has a profound influence on the magnitude of major lymphoid subsets within healthy individuals." Clin Exp Immunol **155**(3): 423-432.
Chiu, W. K., M. Fann, et al. (2006). "Generation and growth of CD28nullCD8+ memory T cells mediated by IL-15 and its induced cytokines." J Immunol **177**(11): 7802-7810.
Chu, C. Q., M. Field, et al. (1992). "Detection of cytokines at the site of tuberculin-induced delayed-type hypersensitivity in man." Clin Exp Immunol **90**(3): 522-529.
Clark, R. and T. Kupper (2005). "Old meets new: the interaction between innate and adaptive immunity." J Invest Dermatol **125**(4): 629-637.
Clark, R. A., B. Chong, et al. (2006). "The vast majority of CLA+ T cells are resident in normal skin." J Immunol **176**(7): 4431-4439.
Clark, R. A., S. J. Huang, et al. (2008). "Human squamous cell carcinomas evade the immune response by down-regulation of vascular E-selectin and recruitment of regulatory T cells." J Exp Med **205**(10): 2221-2234.
Clark, R. A. and T. S. Kupper (2007). "IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin." Blood **109**(1): 194-202.
Clark, R. A., R. Watanabe, et al. (2012). "Skin effector memory T cells do not recirculate and provide immune protection in alemtuzumab-treated CTCL patients." Sci Transl Med **4**(117): 117ra117.
Cohen, N. R., S. Garg, et al. (2009). "Antigen Presentation by CD1 Lipids, T Cells, and NKT Cells in Microbial Immunity." Adv Immunol **102**: 1-94.
Cohrs, R. J., S. K. Mehta, et al. (2008). "Asymptomatic reactivation and shed of infectious varicella zoster virus in astronauts." J Med Virol **80**(6): 1116-1122.
Collin, M., N. McGovern, et al. (2013). "Human dendritic cell subsets." Immunology **140**(1): 22-30.
Colotta, F., F. Re, et al. (1992). "Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products." Blood **80**(8): 2012-2020.

Croft, M., L. M. Bradley, et al. (1994). "Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells." J Immunol **152**(6): 2675-2685.
Cyster, J. G. and S. R. Schwab (2012). "Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs." Annu Rev Immunol **30**: 69-94.
Dancey, J. T., K. A. Deubelbeiss, et al. (1976). "Neutrophil kinetics in man." J Clin Invest **58**(3): 705-715.
de Saint-Vis, B., J. Vincent, et al. (1998). "A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment." Immunity **9**(3): 325-336.
Deaglio, S., K. M. Dwyer, et al. (2007). "Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression." J Exp Med **204**(6): 1257-1265.
Debes, G. F., C. N. Arnold, et al. (2005). "Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues." Nat Immunol **6**(9): 889-894.
Debrus, S., C. Sadzot-Delvaux, et al. (1995). "Varicella-zoster virus gene 63 encodes an immediate-early protein that is abundantly expressed during latency." J Virol **69**(5): 3240-3245.
Doebis, C., K. Siegmund, et al. (2008). "Cellular players and role of selectin ligands in leukocyte recruitment in a T-cell-initiated delayed-type hypersensitivity reaction." Am J Pathol **173**(4): 1067-1076.
Donahue, J. G., P. W. Choo, et al. (1995). "The incidence of herpes zoster." Arch Intern Med **155**(15): 1605-1609.
Dudda, J. C., N. Perdue, et al. (2008). "Foxp3+ regulatory T cells maintain immune homeostasis in the skin." J Exp Med **205**(7): 1559-1565.
Duffy, D., H. Perrin, et al. (2012). "Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells." Immunity **37**(5): 917-929.
Dunne, P. J., L. Belaramani, et al. (2005). "Quiescence and functional reprogramming of Epstein-Barr virus (EBV)-specific CD8+ T cells during persistent infection." Blood **106**(2): 558-565.

Dzionek, A., A. Fuchs, et al. (2000). "BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood." J Immunol **165**(11): 6037-6046.
Ebner, S., Z. Ehammer, et al. (2004). "Expression of C-type lectin receptors by subsets of dendritic cells in human skin." Int Immunol **16**(6): 877-887.
Effros, R. B. and G. Pawelec (1997). "Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion?" Immunol Today **18**(9): 450-454.
Elbe, A., C. A. Foster, et al. (1996). "T-cell receptor alpha beta and gamma delta T cells in rat and human skin--are they equivalent?" Semin Immunol **8**(6): 341-349.
Fagiolo, U., A. Cossarizza, et al. (1993). "Increased cytokine production in mononuclear cells of healthy elderly people." Eur J Immunol **23**(9): 2375-2378.
Fagnoni, F. F., R. Vescovini, et al. (1996). "Expansion of cytotoxic CD8+ CD28- T cells in healthy ageing people, including centenarians." Immunology **88**(4): 501-507.
Fahlen, L., S. Read, et al. (2005). "T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells." J Exp Med **201**(5): 737-746.
Fallarino, F., U. Grohmann, et al. (2003). "Modulation of tryptophan catabolism by regulatory T cells." Nat Immunol **4**(12): 1206-1212.
Fitzhugh, D. J., S. Naik, et al. (2000). "Cutting edge: C-C chemokine receptor 6 is essential for arrest of a subset of memory T cells on activated dermal microvascular endothelial cells under physiologic flow conditions in vitro." J Immunol **165**(12): 6677-6681.
Flacher, V., C. H. Tripp, et al. (2012). "Skin langerin+ dendritic cells transport intradermally injected anti-DEC-205 antibodies but are not essential for subsequent cytotoxic CD8+ T cell responses." J Immunol **188**(5): 2146-2155.
Flacher, V., C. H. Tripp, et al. (2010). "Epidermal Langerhans cells rapidly capture and present antigens from C-type lectin-targeting antibodies deposited in the dermis." J Invest Dermatol **130**(3): 755-762.

Fletcher, J. M., M. Vukmanovic-Stejic, et al. (2005). "Cytomegalovirus-specific CD4+ T cells in healthy carriers are continuously driven to replicative exhaustion." J Immunol **175**(12): 8218-8225.
Floess, S., J. Freyer, et al. (2007). "Epigenetic control of the foxp3 locus in regulatory T cells." PLoS Biol **5**(2): e38.
Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nat Immunol **4**(4): 330-336.
Fontenot, J. D., J. P. Rasmussen, et al. (2005). "Regulatory T cell lineage specification by the forkhead transcription factor foxp3." Immunity **22**(3): 329-341.
Fortin, C. F., A. Larbi, et al. (2007). "GM-CSF activates the Jak/STAT pathway to rescue polymorphonuclear neutrophils from spontaneous apoptosis in young but not elderly individuals." Biogerontology **8**(2): 173-187.
Franceschi, C., M. Capri, et al. (2007). "Inflammaging and anti-inflammaging: a systemic perspective on aging and longevity emerged from studies in humans." Mech Ageing Dev **128**(1): 92-105.
Fuhlbrigge, R. C., J. D. Kieffer, et al. (1997). "Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells." Nature **389**(6654): 978-981.
Gavin, M. A., T. R. Torgerson, et al. (2006). "Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development." Proc Natl Acad Sci U S A **103**(17): 6659-6664.
Gebhardt, T., L. M. Wakim, et al. (2009). "Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus." Nat Immunol **10**(5): 524-530.
Gebhardt, T., P. G. Whitney, et al. (2011). "Different patterns of peripheral migration by memory CD4+ and CD8+ T cells." Nature **477**(7363): 216-219.
Geginat, J., A. Lanzavecchia, et al. (2003). "Proliferation and differentiation potential of human CD8+ memory T-cell subsets in response to antigen or homeostatic cytokines." Blood **101**(11): 4260-4266.
Geijtenbeek, T. B., D. J. Krooshoop, et al. (2000). "DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking." Nat Immunol **1**(4): 353-357.

Geppert, T. D. and P. E. Lipsky (1989). "Antigen presentation at the inflammatory site." Crit Rev Immunol **9**(4): 313-362.
Germain, R. N. and I. Stefanova (1999). "The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation." Annu Rev Immunol **17**: 467-522.
Ghani, S., M. Feuerer, et al. (2009). "T cells as pioneers: antigen-specific T cells condition inflamed sites for high-rate antigen-non-specific effector cell recruitment." Immunology **128**(1 Suppl): e870-880.
Gibbs, J. H., J. Ferguson, et al. (1984). "Histometric study of the localisation of lymphocyte subsets and accessory cells in human Mantoux reactions." J Clin Pathol **37**(11): 1227-1234.
Gilliet, M., W. Cao, et al. (2008). "Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases." Nat Rev Immunol **8**(8): 594-606.
Gillis, S., R. Kozak, et al. (1981). "Immunological studies of aging. Decreased production of and response to T cell growth factor by lymphocytes from aged humans." J Clin Invest **67**(4): 937-942.
Ginhoux, F., F. Tacke, et al. (2006). "Langerhans cells arise from monocytes in vivo." Nat Immunol **7**(3): 265-273.
Giunta, S. (2008). "Exploring the complex relations between inflammation and aging (inflamm-aging): anti-inflamm-aging remodelling of inflamm- aging, from robustness to frailty." Inflamm Res **57**(12): 558-563.
Goodarzi, H., J. Trowbridge, et al. (2007). "Innate immunity: a cutaneous perspective." Clin Rev Allergy Immunol **33**(1-2): 15-26.
Goodwin, K., C. Viboud, et al. (2006). "Antibody response to influenza vaccination in the elderly: a quantitative review." Vaccine **24**(8): 1159-1169.
Goronzy, J. J. and C. M. Weyand (2012). "Immune aging and autoimmunity." Cell Mol Life Sci **69**(10): 1615-1623.
Gowans, J. L. and E. J. Knight (1964). "The Route of Re-Circulation of Lymphocytes in the Rat." Proc R Soc Lond B Biol Sci **159**: 257-282.
Grabbe, S., G. Varga, et al. (2002). "Beta2 integrins are required for skin homing of primed T cells but not for priming naive T cells." J Clin Invest **109**(2): 183-192.

Grewe, M. (2001). "Chronological ageing and photoageing of dendritic cells." Clin Exp Dermatol **26**(7): 608-612.
Griffiths, S. J., N. E. Riddell, et al. (2013). "Age-associated increase of low-avidity cytomegalovirus-specific CD8+ T cells that re-express CD45RA." J Immunol **190**(11): 5363-5372.
Groves, R. W., M. H. Allen, et al. (1995). "Tumour necrosis factor alpha is pro-inflammatory in normal human skin and modulates cutaneous adhesion molecule expression." Br J Dermatol **132**(3): 345-352.
Guttman-Yassky, E., M. A. Lowes, et al. (2007). "Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis." J Allergy Clin Immunol **119**(5): 1210-1217.
Hamann, D., S. Kostense, et al. (1999). "Evidence that human CD8+CD45RA+CD27- cells are induced by antigen and evolve through extensive rounds of division." Int Immunol **11**(7): 1027-1033.
Haniffa, M., A. Shin, et al. (2012). "Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells." Immunity **37**(1): 60-73.
Harley, C. B., A. B. Futcher, et al. (1990). "Telomeres shorten during ageing of human fibroblasts." Nature **345**(6274): 458-460.
Harrington, L. E., R. D. Hatton, et al. (2005). "Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages." Nat Immunol **6**(11): 1123-1132.
Hautz, T., D. Wolfram, et al. (2012). "Mechanisms and mediators of inflammation: potential models for skin rejection and targeted therapy in vascularized composite allotransplantation." Clin Dev Immunol **2012**: 757310.
Hayward, A. R., M. Cosyns, et al. (1998). "Cytokine production in varicella-zoster virus-stimulated cultures of human blood lymphocytes." J Infect Dis **178 Suppl 1**: S95-98.
Henson, S. M., N. E. Riddell, et al. (2012). "Properties of end-stage human T cells defined by CD45RA re-expression." Curr Opin Immunol **24**(4): 476-481.
Hirahara, K., L. Liu, et al. (2006). "The majority of human peripheral blood CD4+CD25highFoxp3+ regulatory T cells bear functional skin-homing receptors." J Immunol **177**(7): 4488-4494.

Hsieh, C. S., H. M. Lee, et al. (2012). "Selection of regulatory T cells in the thymus." Nat Rev Immunol **12**(3): 157-167.
Huang, H., W. Dawicki, et al. (2010). "Tolerogenic dendritic cells induce CD4+CD25hiFoxp3+ regulatory T cell differentiation from CD4+CD25-/loFoxp3- effector T cells." J Immunol **185**(9): 5003-5010.
Huang, S., S. Apasov, et al. (1997). "Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion." Blood **90**(4): 1600-1610.
Huang, Y. P., J. C. Pechere, et al. (1992). "In vivo T cell activation, in vitro defective IL-2 secretion, and response to influenza vaccination in elderly women." J Immunol **148**(3): 715-722.
Hwang, J. M., J. Yamanouchi, et al. (2004). "A critical temporal window for selectin-dependent CD4+ lymphocyte homing and initiation of late-phase inflammation in contact sensitivity." J Exp Med **199**(9): 1223-1234.
Islam, S. A., D. S. Chang, et al. (2011). "Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells." Nat Immunol **12**(2): 167-177.
Itano, A. A. and M. K. Jenkins (2003). "Antigen presentation to naive CD4 T cells in the lymph node." Nat Immunol **4**(8): 733-739.
Itoh, M., T. Takahashi, et al. (1999). "Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance." J Immunol **162**(9): 5317-5326.
Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol **20**: 197-216.
Jiang, X., R. A. Clark, et al. (2012). "Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity." Nature **483**(7388): 227-231.
Jing, Y., E. Shaheen, et al. (2009). "Aging is associated with a numerical and functional decline in plasmacytoid dendritic cells, whereas myeloid dendritic cells are relatively unaltered in human peripheral blood." Hum Immunol **70**(10): 777-784.

Jones, L., A. P. Black, et al. (2006). "Persistent high frequencies of varicella-zoster virus ORF4 protein-specific CD4+ T cells after primary infection." J Virol **80**(19): 9772-9778.
Jordan, M. S., A. Boesteanu, et al. (2001). "Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide." Nat Immunol **2**(4): 301-306.
Kamiya, H., T. Ihara, et al. (1977). "Diagnostic skin test reactions with varicella virus antigen and clinical application of the test." J Infect Dis **136**(6): 784-788.
Kapsenberg, M. L. (2003). "Dendritic-cell control of pathogen-driven T-cell polarization." Nat Rev Immunol **3**(12): 984-993.
Kawai, T. and S. Akira (2011). "Toll-like receptors and their crosstalk with other innate receptors in infection and immunity." Immunity **34**(5): 637-650.
Kearley, J., J. E. Barker, et al. (2005). "Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent." J Exp Med **202**(11): 1539-1547.
Khan, N., A. Hislop, et al. (2004). "Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection." J Immunol **173**(12): 7481-7489.
Kiistala, U. (1968). "Suction blister device for separation of viable epidermis from dermis." J Invest Dermatol **50**(2): 129-137.
Kinchington, P. R., D. Bookey, et al. (1995). "The transcriptional regulatory proteins encoded by varicella-zoster virus open reading frames (ORFs) 4 and 63, but not ORF 61, are associated with purified virus particles." J Virol **69**(7): 4274-4282.
King, C., S. G. Tangye, et al. (2008). "T follicular helper (TFH) cells in normal and dysregulated immune responses." Annu Rev Immunol **26**: 741-766.
Kipling, D. and H. J. Cooke (1990). "Hypervariable ultra-long telomeres in mice." Nature **347**(6291): 400-402.
Kobie, J. J., P. R. Shah, et al. (2006). "T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine." J Immunol **177**(10): 6780-6786.

Koch, S., A. Larbi, et al. (2008). "Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people." Immun Ageing **5**: 6.
Kolaczkowska, E. and P. Kubes (2013). "Neutrophil recruitment and function in health and inflammation." Nat Rev Immunol **13**(3): 159-175.
Kolodkin, A. L., D. V. Levengood, et al. (1997). "Neuropilin is a semaphorin III receptor." Cell **90**(4): 753-762.
Kong, K. F., K. Delroux, et al. (2008). "Dysregulation of TLR3 impairs the innate immune response to West Nile virus in the elderly." J Virol **82**(15): 7613-7623.
Kono, H. and K. L. Rock (2008). "How dying cells alert the immune system to danger." Nat Rev Immunol **8**(4): 279-289.
Koschella, M., D. Voehringer, et al. (2004). "CD40 ligation in vivo induces bystander proliferation of memory phenotype CD8 T cells." J Immunol **172**(8): 4804-4811.
Kupper, T. S. and R. C. Fuhlbrigge (2004). "Immune surveillance in the skin: mechanisms and clinical consequences." Nat Rev Immunol **4**(3): 211-222.
Lages, C. S., I. Suffia, et al. (2008). "Functional regulatory T cells accumulate in aged hosts and promote chronic infectious disease reactivation." J Immunol **181**(3): 1835-1848.
Lanzavecchia, A. and F. Sallusto (2000). "Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells." Science **290**(5489): 92-97.
Lee, K. H., K. Y. Chung, et al. (1995). "Memory T lymphocytes' adherence to interferon gamma-activated human dermal microvascular endothelial cells via E-selectin." J Dermatol Sci **10**(2): 166-175.
Levin, M. J. (2012). "Immune senescence and vaccines to prevent herpes zoster in older persons." Curr Opin Immunol **24**(4): 494-500.
Levin, M. J., M. N. Oxman, et al. (2008). "Varicella-zoster virus-specific immune responses in elderly recipients of a herpes zoster vaccine." J Infect Dis **197**(6): 825-835.
Ley, K., C. Laudanna, et al. (2007). "Getting to the site of inflammation: the leukocyte adhesion cascade updated." Nat Rev Immunol **7**(9): 678-689.

Liang, F., E. Bond, et al. (2013). "Dendritic cell recruitment in response to skin antigen tests in HIV-1-infected individuals correlates with the level of T-cell infiltration." AIDS **27**(7): 1071-1080.
Libri, V., R. I. Azevedo, et al. (2011). "Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4+CD45RA+CD27+ T cells: the potential involvement of interleukin-7 in this process." Immunology **132**(3): 326-339.
Ligthart, G. J., J. X. Corberand, et al. (1984). "Admission criteria for immunogerontological studies in man: the SENIEUR protocol." Mech Ageing Dev **28**(1): 47-55.
Linton, P. J., L. Haynes, et al. (1996). "Antigen-independent changes in naive CD4 T cells with aging." J Exp Med **184**(5): 1891-1900.
Liu, L., R. C. Fuhlbrigge, et al. (2006). "Dynamic programming of CD8+ T cell trafficking after live viral immunization." Immunity **25**(3): 511-520.
Liu, W., A. L. Putnam, et al. (2006). "CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells." J Exp Med **203**(7): 1701-1711.
Lowes, M. A., F. Chamian, et al. (2005). "Increase in TNF-alpha and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a)." Proc Natl Acad Sci U S A **102**(52): 19057-19062.
Mahalingam, R., M. Wellish, et al. (1996). "Expression of protein encoded by varicella-zoster virus open reading frame 63 in latently infected human ganglionic neurons." Proc Natl Acad Sci U S A **93**(5): 2122-2124.
Mahbub, S., C. R. Deburghgraeve, et al. (2012). "Advanced age impairs macrophage polarization." J Interferon Cytokine Res **32**(1): 18-26.
Maini, M. K., M. V. Soares, et al. (1999). "Virus-induced CD8+ T cell clonal expansion is associated with telomerase up-regulation and telomere length preservation: a mechanism for rescue from replicative senescence." J Immunol **162**(8): 4521-4526.
Malavige, G. N., L. Jones, et al. (2008). "Varicella zoster virus glycoprotein E-specific CD4+ T cells show evidence of recent activation and effector differentiation, consistent with frequent exposure to replicative cycle antigens in healthy immune donors." Clin Exp Immunol **152**(3): 522-531.

Mallon, E., S. Powell, et al. (1997). "Evidence for altered cell-mediated immunity in postmastectomy lymphoedema." Br J Dermatol **137**(6): 928-933.
Mandapathil, M., S. Lang, et al. (2009). "Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression." J Immunol Methods **346**(1-2): 55-63.
Marrie, T. J., S. Johnson, et al. (1988). "Cell-mediated immunity of healthy adult Nova Scotians in various age groups compared with nursing home and hospitalized senior citizens." J Allergy Clin Immunol **81**(5 Pt 1): 836-843.
Martinez, F. O. (2011). "Regulators of macrophage activation." Eur J Immunol **41**(6): 1531-1534.
Marzo, A. L., K. D. Klonowski, et al. (2005). "Initial T cell frequency dictates memory CD8+ T cell lineage commitment." Nat Immunol **6**(8): 793-799.
Masopust, D., D. Choo, et al. (2010). "Dynamic T cell migration program provides resident memory within intestinal epithelium." J Exp Med **207**(3): 553-564.
Masopust, D. and J. M. Schenkel (2013). "The integration of T cell migration, differentiation and function." Nat Rev Immunol **13**(5): 309-320.
Masopust, D., V. Vezys, et al. (2001). "Preferential localization of effector memory cells in nonlymphoid tissue." Science **291**(5512): 2413-2417.
Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-305.
Maynard, C. L., L. E. Harrington, et al. (2007). "Regulatory T cells expressing interleukin 10 develop from Foxp3+ and Foxp3- precursor cells in the absence of interleukin 10." Nat Immunol **8**(9): 931-941.
McLachlan, J. B., D. M. Catron, et al. (2009). "Dendritic cell antigen presentation drives simultaneous cytokine production by effector and regulatory T cells in inflamed skin." Immunity **30**(2): 277-288.
Medzhitov, R. and C. A. Janeway, Jr. (2002). "Decoding the patterns of self and nonself by the innate immune system." Science **296**(5566): 298-300.
Merad, M., M. G. Manz, et al. (2002). "Langerhans cells renew in the skin throughout life under steady-state conditions." Nat Immunol **3**(12): 1135-1141.
Mestas, J. and C. C. Hughes (2004). "Of mice and not men: differences between mouse and human immunology." J Immunol **172**(5): 2731-2738.

Miller, L. S. and R. L. Modlin (2007). "Toll-like receptors in the skin." Semin Immunopathol **29**(1): 15-26.
Ming, W. J., L. Bersani, et al. (1987). "Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes." J Immunol **138**(5): 1469-1474.
Miyara, M., Y. Yoshioka, et al. (2009). "Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor." Immunity **30**(6): 899-911.
Mizukawa, Y., Y. Yamazaki, et al. (2008). "In vivo dynamics of intraepidermal CD8+ T cells and CD4+ T cells during the evolution of fixed drug eruption." Br J Dermatol **158**(6): 1230-1238.
Moesgaard, F., M. Lykkegaard Nielsen, et al. (1987). "Cell-mediated immunity assessed by skin testing (Multitest). I. Normal values in healthy Danish adults." Allergy **42**(8): 591-596.
Mosmann, T. R., H. Cherwinski, et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-2357.
Mueller, S. N., T. Gebhardt, et al. (2013). "Memory T cell subsets, migration patterns, and tissue residence." Annu Rev Immunol **31**: 137-161.
Mysliwska, J., E. Bryl, et al. (1999). "The upregulation of TNF alpha production is not a generalised phenomenon in the elderly between their sixth and seventh decades of life." Mech Ageing Dev **107**(1): 1-14.
Nathan, C. F., H. W. Murray, et al. (1983). "Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity." J Exp Med **158**(3): 670-689.
Nepom, G. T. (2012). "MHC class II tetramers." J Immunol **188**(6): 2477-2482.
Nestle, F. O., C. Conrad, et al. (2005). "Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production." J Exp Med **202**(1): 135-143.
Nikkels, A. F., S. Debrus, et al. (1995). "Immunohistochemical identification of varicella-zoster virus gene 63-encoded protein (IE63) and late (gE) protein on smears and cutaneous biopsies: implications for diagnostic use." J Med Virol **47**(4): 342-347.

O'Garra, A., L. M. McEvoy, et al. (1998). "T-cell subsets: chemokine receptors guide the way." Curr Biol **8**(18): R646-649.
O'Gorman, W. E., H. Dooms, et al. (2009). "The initial phase of an immune response functions to activate regulatory T cells." J Immunol **183**(1): 332-339.
Ohkura, N., M. Hamaguchi, et al. (2012). "T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development." Immunity **37**(5): 785-799.
Ohkura, N., Y. Kitagawa, et al. (2013). "Development and maintenance of regulatory T cells." Immunity **38**(3): 414-423.
Okuno, Y., Y. Takao, et al. (2013). "Assessment of skin test with varicella-zoster virus antigen for predicting the risk of herpes zoster." Epidemiol Infect **141**(4): 706-713.
Onishi, Y., Z. Fehervari, et al. (2008). "Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation." Proc Natl Acad Sci U S A **105**(29): 10113-10118.
Ortiz-Suarez, A. and R. A. Miller (2002). "A subset of CD8 memory T cells from old mice have high levels of CD28 and produce IFN-gamma." Clin Immunol **104**(3): 282-292.
Pacholczyk, R., J. Kern, et al. (2007). "Nonself-antigens are the cognate specificities of Foxp3+ regulatory T cells." Immunity **27**(3): 493-504.
Panda, A., A. Arjona, et al. (2009). "Human innate immunosenescence: causes and consequences for immunity in old age." Trends Immunol **30**(7): 325-333.
Park, H., Z. Li, et al. (2005). "A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17." Nat Immunol **6**(11): 1133-1141.
Patterson-Bartlett, J., M. J. Levin, et al. (2007). "Phenotypic and functional characterization of ex vivo T cell responses to the live attenuated herpes zoster vaccine." Vaccine **25**(41): 7087-7093.
Peters, N. C., J. G. Egen, et al. (2008). "In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies." Science **321**(5891): 970-974.

Picker, L. J. and E. C. Butcher (1992). "Physiological and molecular mechanisms of lymphocyte homing." Annu Rev Immunol **10**: 561-591.
Picker, L. J., T. K. Kishimoto, et al. (1991). "ELAM-1 is an adhesion molecule for skin-homing T cells." Nature **349**(6312): 796-799.
Pillay, J., V. M. Kamp, et al. (2012). "A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1." J Clin Invest **122**(1): 327-336.
Platt, J. L., B. W. Grant, et al. (1983). "Immune cell populations in cutaneous delayed-type hypersensitivity." J Exp Med **158**(4): 1227-1242.
Plunkett, F. J., M. V. Soares, et al. (2001). "The flow cytometric analysis of telomere length in antigen-specific CD8+ T cells during acute Epstein-Barr virus infection." Blood **97**(3): 700-707.
Poulter, L. W., G. J. Seymour, et al. (1982). "Immunohistological analysis of delayed-type hypersensitivity in man." Cell Immunol **74**(2): 358-369.
Quinlivan, M. L., K. Ayres, et al. (2007). "Effect of viral load on the outcome of herpes zoster." J Clin Microbiol **45**(12): 3909-3914.
Reiss, Y., A. E. Proudfoot, et al. (2001). "CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin." J Exp Med **194**(10): 1541-1547.
Riley, J. L. and C. H. June (2005). "The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation." Blood **105**(1): 13-21.
Rink, L., I. Cakman, et al. (1998). "Altered cytokine production in the elderly." Mech Ageing Dev **102**(2-3): 199-209.
Rogers, P. R., C. Dubey, et al. (2000). "Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen." J Immunol **164**(5): 2338-2346.
Romero, P., A. Zippelius, et al. (2007). "Four functionally distinct populations of human effector-memory CD8+ T lymphocytes." J Immunol **178**(7): 4112-4119.
Rosen, S. D. (2004). "Ligands for L-selectin: homing, inflammation, and beyond." Annu Rev Immunol **22**: 129-156.
Rossi, D. and A. Zlotnik (2000). "The biology of chemokines and their receptors." Annu Rev Immunol **18**: 217-242.

Rossing, N. and A. M. Worm (1981). "Interstitial fluid: exchange of macromolecules between plasma and skin interstitium." Clin Physiol **1**(3): 275-284.
Rubtsov, Y. P., J. P. Rasmussen, et al. (2008). "Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces." Immunity **28**(4): 546-558.
Rufer, N., T. H. Brummendorf, et al. (1999). "Telomere fluorescence measurements in granulocytes and T lymphocyte subsets point to a high turnover of hematopoietic stem cells and memory T cells in early childhood." J Exp Med **190**(2): 157-167.
Sadaoka, K., S. Okamoto, et al. (2008). "Measurement of varicella-zoster virus (VZV)-specific cell-mediated immunity: comparison between VZV skin test and interferon-gamma enzyme-linked immunospot assay." J Infect Dis **198**(9): 1327-1333.
Saeki, H. and K. Tamaki (2006). "Thymus and activation regulated chemokine (TARC)/CCL17 and skin diseases." J Dermatol Sci **43**(2): 75-84.
Sakaguchi, S. (2004). "Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses." Annu Rev Immunol **22**: 531-562.
Sakaguchi, S., N. Sakaguchi, et al. (1995). "Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases." J Immunol **155**(3): 1151-1164.
Sakaguchi, S., T. Yamaguchi, et al. (2008). "Regulatory T cells and immune tolerance." Cell **133**(5): 775-787.
Sallusto, F., J. Geginat, et al. (2004). "Central memory and effector memory T cell subsets: function, generation, and maintenance." Annu Rev Immunol **22**: 745-763.
Sallusto, F., D. Lenig, et al. (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature **401**(6754): 708-712.
Salmi, M. and S. Jalkanen (2005). "Lymphocyte homing to the gut: attraction, adhesion, and commitment." Immunol Rev **206**: 100-113.

Salmon, M., D. Pilling, et al. (1994). "The progressive differentiation of primed T cells is associated with an increasing susceptibility to apoptosis." Eur J Immunol **24**(4): 892-899.
Sauce, D., M. Larsen, et al. (2007). "IL-7R alpha versus CCR7 and CD45 as markers of virus-specific CD8+ T cell differentiation: contrasting pictures in blood and tonsillar lymphoid tissue." J Infect Dis **195**(2): 268-278.
Schenkel, J. M., K. A. Fraser, et al. (2013). "Sensing and alarm function of resident memory CD8(+) T cells." Nat Immunol **14**(5): 509-513.
Schroder, J. M. (1995). "Cytokine networks in the skin." J Invest Dermatol **105**(1 Suppl): 20S-24S.
Schuler, P. J., M. Harasymczuk, et al. (2011). "Separation of human CD4+CD39+ T cells by magnetic beads reveals two phenotypically and functionally different subsets." J Immunol Methods **369**(1-2): 59-68.
Schwarz, A., F. Navid, et al. (2011). "In vivo reprogramming of UV radiation-induced regulatory T-cell migration to inhibit the elicitation of contact hypersensitivity." J Allergy Clin Immunol **128**(4): 826-833.
Schwarz, T. (2008). "25 years of UV-induced immunosuppression mediated by T cells-from disregarded T suppressor cells to highly respected regulatory T cells." Photochem Photobiol **84**(1): 10-18.
Schwele, S., A. M. Fischer, et al. (2012). "Cytomegalovirus-specific regulatory and effector T cells share TCR clonality--possible relation to repetitive CMV infections." Am J Transplant **12**(3): 669-681.
Seddiki, N., B. Santner-Nanan, et al. (2006). "Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells." J Exp Med **203**(7): 1693-1700.
Seneschal, J., R. A. Clark, et al. (2012). "Human epidermal Langerhans cells maintain immune homeostasis in skin by activating skin resident regulatory T cells." Immunity **36**(5): 873-884.
Shamri, R., V. Grabovsky, et al. (2005). "Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines." Nat Immunol **6**(5): 497-506.

Shiow, L. R., D. B. Rosen, et al. (2006). "CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs." Nature **440**(7083): 540-544.
Siegal, F. P., N. Kadowaki, et al. (1999). "The nature of the principal type 1 interferon-producing cells in human blood." Science **284**(5421): 1835-1837.
Sigmundsdottir, H. and E. C. Butcher (2008). "Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking." Nat Immunol **9**(9): 981-987.
Soker, S., S. Takashima, et al. (1998). "Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor." Cell **92**(6): 735-745.
Sparber, F., C. H. Tripp, et al. (2010). "Langerhans cells and dermal dendritic cells capture protein antigens in the skin: possible targets for vaccination through the skin." Immunobiology **215**(9-10): 770-779.
Sridharan, A., M. Esposo, et al. (2011). "Age-associated impaired plasmacytoid dendritic cell functions lead to decreased CD4 and CD8 T cell immunity." Age (Dordr) **33**(3): 363-376.
Srinivas, U., M. Larsson, et al. (1993). "E-selectin involvement in in vitro adhesion of blood dendritic cells to human umbilical cord endothelial cells." Scand J Immunol **38**(3): 273-278.
Steinmann, G. G., B. Klaus, et al. (1985). "The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study." Scand J Immunol **22**(5): 563-575.
Strauss, L., C. Bergmann, et al. (2007). "A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment." Clin Cancer Res **13**(15 Pt 1): 4345-4354.
Strutt, T. M., K. K. McKinstry, et al. (2010). "Memory CD4+ T cells induce innate responses independently of pathogen." Nat Med **16**(5): 558-564, 551p following 564.
Surh, C. D. and J. Sprent (2008). "Homeostasis of naive and memory T cells." Immunity **29**(6): 848-862.

Taams, L. S., J. M. van Amelsfort, et al. (2005). "Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells." Hum Immunol **66**(3): 222-230.
Takahashi, M., H. Kamiya, et al. (2001). "Immunization of the elderly to boost immunity against varicella-zoster virus (VZV) as assessed by VZV skin test reaction." Arch Virol Suppl(17): 161-172.
Takahashi, M., S. Okada, et al. (2003). "Enhancement of immunity against VZV by giving live varicella vaccine to the elderly assessed by VZV skin test and IAHA, gpELISA antibody assay." Vaccine **21**(25-26): 3845-3853.
Takahashi, T., Y. Kuniyasu, et al. (1998). "Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state." Int Immunol **10**(12): 1969-1980.
Teijaro, J. R., D. Turner, et al. (2011). "Cutting edge: Tissue-retentive lung memory CD4 T cells mediate optimal protection to respiratory virus infection." J Immunol **187**(11): 5510-5514.
Thompson, J. S., D. R. Wekstein, et al. (1984). "The immune status of healthy centenarians." J Am Geriatr Soc **32**(4): 274-281.
Thornton, A. M., P. E. Korty, et al. (2010). "Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells." J Immunol **184**(7): 3433-3441.
Thornton, A. M. and E. M. Shevach (1998). "CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production." J Exp Med **188**(2): 287-296.
Tiemessen, M. M., A. L. Jagger, et al. (2007). "CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages." Proc Natl Acad Sci U S A **104**(49): 19446-19451.
Tough, D. F., P. Borrow, et al. (1996). "Induction of bystander T cell proliferation by viruses and type I interferon in vivo." Science **272**(5270): 1947-1950.

Tough, D. F., X. Zhang, et al. (2001). "An IFN-gamma-dependent pathway controls stimulation of memory phenotype CD8+ T cell turnover in vivo by IL-12, IL-18, and IFN-gamma." J Immunol **166**(10): 6007-6011.
Trinchieri, G. (1995). "The two faces of interleukin 12: a pro-inflammatory cytokine and a key immunoregulatory molecule produced by antigen-presenting cells." Ciba Found Symp **195**: 203-214; discussion 214-220.
Trowbridge, I. S. and M. L. Thomas (1994). "CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development." Annu Rev Immunol **12**: 85-116.
Trzonkowski, P., E. Szmit, et al. (2006). "CD4+CD25+ T regulatory cells inhibit cytotoxic activity of CTL and NK cells in humans-impact of immunosenescence." Clin Immunol **119**(3): 307-316.
Tsuda, Y., H. Takahashi, et al. (2004). "Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus." Immunity **21**(2): 215-226.
Underhill, D. M., M. Bassetti, et al. (1999). "Dynamic interactions of macrophages with T cells during antigen presentation." J Exp Med **190**(12): 1909-1914.
Valenzuela, H. F. and R. B. Effros (2002). "Divergent telomerase and CD28 expression patterns in human CD4 and CD8 T cells following repeated encounters with the same antigenic stimulus." Clin Immunol **105**(2): 117-125.
Valmori, D., A. Merlo, et al. (2005). "A peripheral circulating compartment of natural naive CD4 Tregs." J Clin Invest **115**(7): 1953-1962.
van Duin, D., H. G. Allore, et al. (2007). "Prevaccine determination of the expression of costimulatory B7 molecules in activated monocytes predicts influenza vaccine responses in young and older adults." J Infect Dis **195**(11): 1590-1597.
van Duin, D., S. Mohanty, et al. (2007). "Age-associated defect in human TLR-1/2 function." J Immunol **178**(2): 970-975.
Van Dyken, S. J. and R. M. Locksley (2013). "Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease." Annu Rev Immunol **31**: 317-343.

van Furth, R. and Z. A. Cohn (1968). "The origin and kinetics of mononuclear phagocytes." J Exp Med **128**(3): 415-435.
Vasto, S., G. Candore, et al. (2007). "Inflammatory networks in ageing, age-related diseases and longevity." Mech Ageing Dev **128**(1): 83-91.
Veiga-Fernandes, H., U. Walter, et al. (2000). "Response of naive and memory CD8+ T cells to antigen stimulation in vivo." Nat Immunol **1**(1): 47-53.
Verhagen, J. and D. C. Wraith (2010). "Comment on "Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells"." J Immunol **185**(12): 7129; author reply 7130.
Verjans, G. M., R. Q. Hintzen, et al. (2007). "Selective retention of herpes simplex virus-specific T cells in latently infected human trigeminal ganglia." Proc Natl Acad Sci U S A **104**(9): 3496-3501.
Vignali, D. A., L. W. Collison, et al. (2008). "How regulatory T cells work." Nat Rev Immunol **8**(7): 523-532.
von Andrian, U. H. and C. R. Mackay (2000). "T-cell function and migration. Two sides of the same coin." N Engl J Med **343**(14): 1020-1034.
Vukmanovic-Stejic, M., E. Agius, et al. (2008). "The kinetics of CD4+Foxp3+ T cell accumulation during a human cutaneous antigen-specific memory response in vivo." J Clin Invest **118**(11): 3639-3650.
Vukmanovic-Stejic, M., J. R. Reed, et al. (2006). "Mantoux Test as a model for a secondary immune response in humans." Immunol Lett **107**(2): 93-101.
Vukmanovic-Stejic, M., Y. Zhang, et al. (2006). "Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo." J Clin Invest **116**(9): 2423-2433.
Wakim, L. M., T. Gebhardt, et al. (2008). "Cutting edge: local recall responses by memory T cells newly recruited to peripheral nonlymphoid tissues." J Immunol **181**(9): 5837-5841.
Wakim, L. M., J. Waithman, et al. (2008). "Dendritic cell-induced memory T cell activation in nonlymphoid tissues." Science **319**(5860): 198-202.
Wakim, L. M., A. Woodward-Davis, et al. (2010). "Memory T cells persisting within the brain after local infection show functional adaptations to their tissue of residence." Proc Natl Acad Sci U S A **107**(42): 17872-17879.

Walker, M. R., B. D. Carson, et al. (2005). "De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+CD25- cells." Proc Natl Acad Sci U S A **102**(11): 4103-4108.
Wammanda, R. D., M. J. Gambo, et al. (2006). "The mantoux test: should we change the site?" Trop Doct **36**(1): 40.
Wang, J., A. Ioan-Facsinay, et al. (2007). "Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells." Eur J Immunol **37**(1): 129-138.
Weinberger, B., D. Herndler-Brandstetter, et al. (2008). "Biology of immune responses to vaccines in elderly persons." Clin Infect Dis **46**(7): 1078-1084.
Weiss, J. M., A. M. Bilate, et al. (2012). "Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells." J Exp Med **209**(10): 1723-1742, S1721.
Weng, N. P. (2006). "Aging of the immune system: how much can the adaptive immune system adapt?" Immunity **24**(5): 495-499.
Weng, N. P., B. L. Levine, et al. (1995). "Human naive and memory T lymphocytes differ in telomeric length and replicative potential." Proc Natl Acad Sci U S A **92**(24): 11091-11094.
Weng, N. P., B. L. Levine, et al. (1996). "Regulated expression of telomerase activity in human T lymphocyte development and activation." J Exp Med **183**(6): 2471-2479.
Wherry, E. J., V. Teichgraber, et al. (2003). "Lineage relationship and protective immunity of memory CD8 T cell subsets." Nat Immunol **4**(3): 225-234.
Wick, G. and B. Grubeck-Loebenstein (1997). "The aging immune system: primary and secondary alterations of immune reactivity in the elderly." Exp Gerontol **32**(4-5): 401-413.
Wilson, E. B., D. H. Yamada, et al. (2013). "Blockade of chronic type I interferon signaling to control persistent LCMV infection." Science **340**(6129): 202-207.
Wong, C. and D. R. Goldstein (2013). "Impact of aging on antigen presentation cell function of dendritic cells." Curr Opin Immunol.
Wurbel, M. A., M. Malissen, et al. (2001). "Mice lacking the CCR9 CC-chemokine receptor show a mild impairment of early T- and B-cell

development and a reduction in T-cell receptor gammadelta(+) gut intraepithelial lymphocytes." Blood **98**(9): 2626-2632.
Wyble, C. W., K. L. Hynes, et al. (1997). "TNF-alpha and IL-1 upregulate membrane-bound and soluble E-selectin through a common pathway." J Surg Res **73**(2): 107-112.
Yadav, M., C. Louvet, et al. (2012). "Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo." J Exp Med **209**(10): 1713-1722, S1711-1719.
Yamaguchi, T., J. B. Wing, et al. (2011). "Two modes of immune suppression by Foxp3(+) regulatory T cells under inflammatory or non-inflammatory conditions." Semin Immunol **23**(6): 424-430.
Yamane, H. and W. E. Paul (2012). "Memory CD4+ T cells: fate determination, positive feedback and plasticity." Cell Mol Life Sci **69**(10): 1577-1583.
Yin, J. and T. A. Ferguson (2009). "Identification of an IFN-gamma-producing neutrophil early in the response to Listeria monocytogenes." J Immunol **182**(11): 7069-7073.
Yokosuka, T., W. Kobayashi, et al. (2010). "Spatiotemporal basis of CTLA-4 costimulatory molecule-mediated negative regulation of T cell activation." Immunity **33**(3): 326-339.
Yoshikawa, T. T. (2000). "Epidemiology and unique aspects of aging and infectious diseases." Clin Infect Dis **30**(6): 931-933.
Zaba, L. C., J. Fuentes-Duculan, et al. (2007). "Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163+FXIIIA+ macrophages." J Clin Invest **117**(9): 2517-2525.
Zaba, L. C., J. G. Krueger, et al. (2009). "Resident and "inflammatory" dendritic cells in human skin." J Invest Dermatol **129**(2): 302-308.
Zhou, R., R. Horai, et al. (2012). "The living eye "disarms" uncommitted autoreactive T cells by converting them to Foxp3(+) regulatory cells following local antigen recognition." J Immunol **188**(4): 1742-1750.